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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No. TSRI 409.1D2
First Inventor or Application Identifier Barbas, et. al.
Title METHODS FOR PRODUCING ANTIBODY LIBRARIES...
Express Mail Label No. EL193834634US

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. ☒ Specification [Total Pages 130]
(preferred arrangement set forth below)
- Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the invention
 - Brief Summary of the invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 2]
4. Oath or Declaration [Total Pages 2]
- a. ☐ Newly executed (original or copy)
- b. ☒ Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
- i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).
5. ☒ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
- a. ☐ Computer Readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 C.F.R. § 3.73(b) Statement (when there is an assignee) ☐ Power of Attorney
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☒ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
14. ☐ * Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired (PTO/SB/09-12)
15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. ☒ Other: Certificate of Express Mail

* NOTE FOR ITEMS 1 & 14: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application No: 08, 931, 645

Prior application information: Examiner M. Tung Group / Art Unit: 1644

18. CORRESPONDENCE ADDRESS

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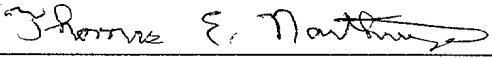
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this PRELIMINARY AMENDMENT and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service Mail Label No. EL193834634US under 37 CFR 1.10 on the date indicated below and is addressed to: Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

	7-5-2000
Thomas E. Northrup, Reg. No. 33,268	Date of Deposit
Applicant: Barbas, et al.)
Serial No.: Unassigned) Group Art Unit: Unassigned
Filed: July 5, 2000) Examiner: Unassigned
Title: METHODS FOR PRODUCING)
ANTIBODY LIBRARIES USING)
UNIVERSAL OR RANDOMIZED)
IMMUNOGLOBULIN LIGHT CHAINS) Our Ref.: TSRI 409.1Div2

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

ATTN: BOX PATENT APPLICATION

Dear Sir:

Prior to examination of the above-identified patent application on the merits, which is a Divisional application of pending prior application U.S. Serial No. 08/931,645 filed under 37 CFR 1.53(b) concurrently herewith, please enter the following amendments.

IN THE CLAIMS

Please cancel claims 2, 3, 6, 7, 9, 10, 13 and 35-37.

REMARKS

Entry of the above amendment is respectfully requested.
Claims 2, 3, 6, 7, 9, 10, 13, and 35-37 have been cancelled.
Claims 1, 4, 5, 8, 11, 12, and 14-34 are now pending. No new
matter has been added.

Respectfully submitted,

7-5-2000

Date

Thomas E. Northrup

Thomas E. Northrup, Reg. No. 33,268

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PATENT

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Thomas E. Northrup
Thomas E. Northrup, Reg. No. 33,268

7-5-2000
Date of Deposit

Applicant: Barbas, et al.)	
)	
Serial No.: Unassigned)	Group Art Unit: Unassigned
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Filed: July 5, 2000)	Examiner: Unassigned
)	
Title: METHODS FOR PRODUCING)	
ANTIBODY LIBRARIES USING)	
UNIVERSAL OR RANDOMIZED)	
IMMUNOGLOBULIN LIGHT CHAINS)	Our Ref.: TSRI 409.1D2
)	

TSRI 409.1

METHODS FOR PRODUCING ANTIBODY LIBRARIES USING
UNIVERSAL OR RANDOMIZED IMMUNOGLOBULIN LIGHT CHAINS

5 This invention was made with government support
under Contract Nos. AI 33292 and CA 27489 by The
National Institutes of Health. The government has
certain rights in the invention.

Cross-Reference to Related Application

10 This application is related to copending
application Serial No. 07/826,623, filed January 27,
1992, Serial No. 07/954,148, filed September 30, 1992,
Serial No. 08/012,566, filed February 2, 1993, and
Serial No. 08/174,674, filed December 28, 1993, the
15 disclosures of which are hereby incorporated by
reference.

Technical Field

20 The present invention relates generally to the
field of protein biochemistry and immunology, and
relates specifically to methods for the preparation of
heterodimeric immunoglobulin molecules containing
heavy and light variable chain polypeptides.

25 Background

Large libraries of wholly or partially synthetic
antibody combining sites, or paratopes, have been
constructed utilizing filamentous phage display
vectors, referred to as phagemids, yielding large
30 libraries of monoclonal antibodies having diverse and
novel immunospecificities. The technology uses a
filamentous phage coat protein membrane anchor domain
as a means for linking gene-product and gene during
the assembly stage of filamentous phage replication,
35 and has been used for the cloning and expression of

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antibodies from combinatorial libraries. Kang et al.,
Proc. Natl. Acad. Sci., USA, 88:4363-4366 (1991).

Combinatorial libraries of antibodies have been
produced using both the cpVIII membrane anchor (Kang
et al., supra) and the cpIII membrane anchor (Barbas
et al., Proc. Natl. Acad. Sci., USA, 88:7978-7982
(1991)).

The diversity of a filamentous phage-based
combinatorial antibody library can be increased by
shuffling of the heavy and light chain genes (Kang et
al., Proc. Natl. Acad. Sci., USA, 88:11120-11123,
1991), by altering the complementarity determining
region 3 (CDR3) of the cloned heavy chain genes of the
library (Barbas et al., Proc. Natl. Acad. Sci., USA,
89:4457-4461, 1992), and by introducing random
mutations into the library by error-prone polymerase
chain reactions (PCR) (Gram et al., Proc. Natl. Acad.
Sci., USA, 89:3576-3580, 1992).

Mutagenesis of proteins has been utilized to
alter the function, and in some cases the binding
specificity, of a protein. Typically, the
mutagenesis is site-directed, and therefore laborious
depending on the systematic choice of mutation to
induce in the protein. See, for example Corey et al.,
J. Amer. Chem. Soc., 114:1784-1790 (1992), in which
rat trypsin were modified by site-directed
mutagenesis. Partial randomization of selected codons
in the thymidine kinase (TK) gene was used as a
mutagenesis procedure to develop variant TK proteins.
Munir et al., J. Biol. Chem., 267:6584-6589 (1992).

There continues to be a need for methods to
increase the repertoire of possible antibody molecules
from which to manipulate useful binding functions,
including heavy chain and light chain immunoglobulin
polypeptides.

Brief Description of the Invention

It has now been discovered that the phagemid display technology can be improved by manipulations of the immunoglobulin light chain to prepare diverse libraries of immunoglobulin specificities. In particular, it is shown that the immunoglobulin light chain variable domain can be randomized in its complementarity determining regions (CDR) by random mutagenesis to yield larger and more diverse libraries of light chains from which to draw novel and useful immunospecificities.

Thus, in one embodiment, the invention describes a method for inducing mutagenesis in a complementarity determining region (CDR) of an immunoglobulin light chain gene for the purpose of producing light chain gene libraries for use in combination with heavy chain genes and gene libraries to produce antibody libraries of diverse and novel immunospecificities. The method comprises amplifying a CDR portion of an immunoglobulin light chain gene by polymerase chain reaction (PCR) using a PCR primer oligonucleotide, where the oligonucleotide has 3' and 5' termini and comprises:

- a) a nucleotide sequence at its 3' terminus capable of hybridizing to a first framework region of an immunoglobulin light chain gene;
- b) a nucleotide sequence at its 5' terminus capable of hybridizing to a second framework region of the immunoglobulin light chain gene; and
- c) a nucleotide sequence between the 3' and 5' termini according to the formula:

$$[NNK]_n,$$

wherein N is independently any nucleotide, K is G or T, and n is 3 to about 24, said 3' and 5' terminal

nucleotide sequences having a length of about 6 to 50 nucleotides. Also contemplated are oligonucleotides having a sequence complementary thereto.

In a preferred embodiment, the invention
5 contemplates the above mutagenesis method that further comprises the steps of:

a) isolating the amplified CDR to form a library of mutagenized immunoglobulin light chain genes;

10 b) expressing the isolated library of mutagenized light chain genes in combination with one or more heavy chain genes to form a combinatorial antibody library of expressed heavy and light chain genes; and

15 c) selecting species of the combinatorial antibody library for the ability to bind a preselected antigen. In one embodiment, the one or more immunoglobulin heavy chain genes can be provided as a library of heavy chain genes as described further
20 herein.

In a related embodiment, the oligonucleotide used in the method can have a nucleotide sequence between the 3' and 5' termini according to the formula:

$$[MNN]_n,$$

25 wherein N is independently any nucleotide, M is A or C, and n is 3 to about 24.

In addition, it is shown in the present invention that particular immunoglobulin light chain variable domain polypeptides are useful as a light chain
30 partner for a large variety of heavy chains, i.e., the light chain forms functional heterodimeric antibody molecules upon association with different heavy chains, demonstrating the ability to function universally as a light chain in the presently
35 described combinatorial libraries.

Thus, in preferred mutagenesis methods, the immunoglobulin variable domain light chain gene includes a sequence having the sequence characteristics selected from the group consisting of light chains shown in SEQ ID NOs 2 and 62 which encode the preferred universal light chain polypeptides described herein.

In a related embodiment, the invention contemplates the direct use of the universal light chain polypeptide gene without diversification by mutagenesis of its CDR domains. Specifically, the invention contemplates a method for producing a heterodimeric immunoglobulin molecule having immunoglobulin variable domain heavy and light chain polypeptides comprising the steps of:

- a) combining an immunoglobulin variable domain light chain gene that includes a sequence having the sequence characteristics of a light chain selected from the group consisting of light chains shown in SEQ ID NO 2 and 62 with one or more immunoglobulin variable domain heavy chain genes to form a combinatorial immunoglobulin heavy and light chain gene library, where the combining comprising operatively linking the light chain gene with one of the heavy chain genes in a vector capable of co-expression of the heavy and light chain genes;
- b) expressing the combinatorial gene library to form a combinatorial antibody library of expressed heavy and light chain polypeptides; and
- c) selecting species of the combinatorial antibody library for the ability to bind a preselected antigen.

Also contemplated are oligonucleotide compositions for use as PCR primers to perform the recited mutageneses.

Brief Description of the Drawings

In the drawings forming a portion of this disclosure:

5 Figure 1 illustrates the structures of hapten conjugates used for selection of the semisynthetic Fab heterodimers of this invention. Conjugate 1 is fluorescein-BSA (Fl-BSA) as described in Example 5B. Conjugates 2 and 3, respectively, S-BSA and C-BSA,
10 were prepared as described in Example 5B.

15 Figure 2 graphically depicts the anti-synthetic hapten conjugate specificity of selected Fab heterodimers by ELISA. The antigens used in the ELISA shown from left to right are the original pC3AP313-specific tetanus toxoid (forward slashed bar), Fl-BSA conjugate (black bar), BSA (horizontal bar), S-BSA conjugate (backward slashed bar) and C-BSA conjugate (white bar). Standard ELISA was performed as described in Example 6A.
20

Detailed Description of the Invention

A. Definitions

25 Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH₂ refers to the free amino group
30 present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR
35

§1.822(b)(2)), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

5	SYMBOL		AMINO ACID
	<u>1-Letter</u>	<u>3-Letter</u>	
	Y	Tyr	tyrosine
	G	Gly	glycine
	F	Phe	phenylalanine
10	M	Met	methionine
	A	Ala	alanine
	S	Ser	serine
	I	Ile	isoleucine
	L	Leu	leucine
15	T	Thr	threonine
	V	Val	valine
	P	Pro	proline
	K	Lys	lysine
	H	His	histidine
20	Q	Gln	glutamine
	E	Glu	glutamic acid
	Z	Glx	Glu and/or Gln
	W	Trp	tryptophan
	R	Arg	arginine
25	D	Asp	aspartic acid
	N	Asn	asparagine
	B	Asx	Asn and/or Asp
	C	Cys	cysteine
	X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by formulae have a left-to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly

defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those listed in 37 CFR 1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to an amino-terminal group such as NH₂ or acetyl or to a carboxy-terminal group such as COOH.

Recombinant DNA (rDNA) Molecule: A DNA molecule produced by operatively linking two DNA segments. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature. rDNA's not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

Vector: A rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to herein as "expression vectors". Particularly important vectors allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

Receptor: A receptor is a molecule, such as a protein, glycoprotein and the like, that can specifically (non-randomly) bind to another molecule.

Antibody: The term antibody in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules

that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v).

Antibody Combining Site: An antibody combining site is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen. The term immunoreact in its various forms means specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

Monoclonal Antibody: A monoclonal antibody in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different epitope, e.g., a bispecific monoclonal antibody. Although historically a monoclonal antibody was produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the present invention.

Fusion Polypeptide: A polypeptide comprised of at least two polypeptides and a linking sequence to operatively link the two polypeptides into one continuous polypeptide. The two polypeptides linked

in a fusion polypeptide are typically derived from two independent sources, and therefore a fusion polypeptide comprises two linked polypeptides not normally found linked in nature.

5 Upstream: In the direction opposite to the direction of DNA transcription, and therefore going from 5' to 3' on the noncodingstrand, or 3' to 5' on the mRNA.

10 Downstream: Further along a DNA sequence in the direction of sequence transcription or read out, that is traveling in a 3'- to 5'-direction along the noncodingstrand of the DNA or 5'- to 3'-direction along the RNA transcript.

15 Cistron: A sequence of nucleotides in a DNA molecule coding for an amino acid residue sequence and including upstream and downstream DNA expression control elements.

20 Leader Polypeptide: A short length of amino acid sequence at the amino end of a polypeptide, which carries or directs the polypeptide through the inner membrane and so ensures its eventual secretion into the periplasmic space and perhaps beyond. The leader sequence peptide is commonly removed before the polypeptide becomes active.

25 Reading Frame: A particular sequence of contiguous nucleotide triplets (codons) employed in translation. The reading frame depends on the location of the translation initiation codon.

30 B. Methods For Producing Antibody Molecules or Libraries of Antibody Molecules

1. General Rationale

35 The present invention utilizes a system for the simultaneous cloning and screening of preselected ligand-binding specificities from gene

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plurality of DNA expression vectors in linear form adapted to form a fusion protein expressing cistron, and (b) subjecting the admixture to ligation conditions for a time period sufficient for the repertoire of genes to become operatively linked (ligated) to the plurality of vectors to form the library.

In this embodiment, the repertoire of polypeptide encoding genes are in the form of double-stranded (ds) DNA and each member of the repertoire has cohesive termini adapted for directional ligation. In addition, the plurality of DNA expression vectors are each linear DNA molecules having upstream and downstream cohesive termini that are (a) adapted for directionally receiving the polypeptide genes in a common reading frame, and (b) operatively linked to respective upstream and downstream translatable DNA sequences. The upstream translatable DNA sequence encodes a secretion signal, preferably a pelB secretion signal, and the downstream translatable DNA sequence encodes a filamentous phage coat protein membrane anchor as described herein for a polypeptide of this invention. The translatable DNA sequences are also operatively linked to respective upstream and downstream DNA expression control sequences as defined for a DNA expression vector described herein.

The library so produced can be utilized for expression and screening of the fusion proteins encoded by the resulting library of cistrons represented in the library by the expression and screening methods described herein.

2. Production of Gene Repertoires

A gene repertoire is a collection of different genes, preferably polypeptide-encoding genes

(polypeptide genes), and may be isolated from natural sources or can be generated artificially. Preferred gene repertoires are comprised of conserved genes. Particularly preferred gene repertoires comprise either or both genes that code for polypeptides that can assemble to form a functional dimeric receptor molecule.

A gene repertoire useful in practicing the present invention contains at least 10^3 , preferably at least 10^4 , more preferably at least 10^5 , and most preferably at least 10^7 different genes. Methods for evaluating the diversity of a repertoire of genes is well known to one skilled in the art.

Preferably, the receptor will be a heterodimeric polypeptide capable of binding a ligand, such as an antibody molecule or immunologically active portion thereof, coded for by one of the members of a family (repertoire) of conserved genes, i.e., genes containing a conserved nucleotide sequence of at least about 10 nucleotides in length.

A gene can be identified as belonging to a repertoire of conserved genes using several methods. For example, an isolated gene may be used as a hybridization probe under low stringency conditions to detect other members of the repertoire of conserved genes present in genomic DNA using the methods described by Southern, J. Mol. Biol., 98:503 (1975). If the gene used as a hybridization probe hybridizes to multiple restriction endonuclease fragments of the genome, that gene is a member of a repertoire of conserved genes.

The present invention relates generally to methods for producing novel antibody molecules by the preparation of diverse libraries of antibodies, and subsequent screening of the libraries for desirable

binding specificities. The method involves the preparation of libraries of heterodimeric immunoglobulin molecules in the form of phagemid libraries using degenerate oligonucleotides and primer extension reactions to incorporate the degeneracies into the CDR regions of the immunoglobulin variable heavy and light chain variable domains, and display of the mutagenized polypeptides on the surface of the phagemid. Thereafter, the display protein is screened for the ability to bind to a preselected antigen.

Furthermore, the libraries of heavy and light chain immunoglobulin-coding genes can be crossed to form random pairings of species of heavy and light chains, yielding higher numbers of unique heterodimers. Such crosses can be conducted in a variety of ways, as described further herein, including (1) crossing a single heavy chain to a library of light chains, (2) crossing a single light chain to a library of heavy chains, (3) crossing a randomized light or heavy chain against a single heavy or light chain, respectively, (4) crossing a randomized light or heavy chain against a heavy or light chain library, respectively, and (5) crossing a randomized light or heavy chain against a randomized heavy or light chain, respectively. Other permutations are also apparent.

By randomized is meant generally to connote the preparation of a library of light (or heavy) chain genes by mutagenesis of one or more CDR regions in the variable domain of a preselected light or heavy chain, as described further herein.

One particularly preferred permutation of the above methods to produce an antibody repertoire is by the use of randomized light chain genes crossed with a heavy chain library, and particularly crossed with a

randomized heavy chain library. Another particularly preferred embodiment is the use of a "universal light chain" selected from the group consisting of light chains shown in SEQ ID NOs 2 and 62 as described further herein as the single light chain in the cross with a heavy chain library. A preferred related embodiment is the use of a randomized universal light chain against a heavy chain or heavy chain library. Other preferred methods are also described herein.

3. Phagemid Display Proteins

The display of the heterodimeric immunoglobulin molecule as a display protein on a phagemid can be accomplished on any of the surface proteins of the filamentous phage particle, although particularly preferred are display proteins comprising gene III or gene VIII protein, as described herein. The use of gene III or gene VIII protein as a display protein on filamentous phage has been extensively described elsewhere herein.

Particularly preferred display proteins are fusions involving the use of the phage particle membrane anchor derived from gene III or gene VIII fused to an immunoglobulin heavy or light chain as described herein. In this embodiment, a polypeptide containing at least one variable domain CDR of an immunoglobulin heavy or light chain is fused to the membrane anchor domain of the phage's gene III or gene VIII protein. Preferably, a complete variable domain is fused, including all the CDR's ,

When using an immunoglobulin heavy or light chain variable region, the fusion protein can include one or more of the complementarity determining regions, CDR1, CDR2 or CDR3. Using the Kabat immunoglobulin amino acid residue sequence position numbering system, the

light chain CDR's are as follows: CDR1 (residues 23-35), CDR2 (residues 49-57), and CDR3 (residues 88-98); and the heavy chain CDR's are as follows: CDR1 (residues 30-36), CDR2 (residues 49-66), and CDR3 (residues 94-103). See, Kabat et al., "Sequences of Proteins of Immunological Interest", 5th ed., NIH, (1991).

When mutagenizing a CDR of an immunoglobulin fusion display protein, some, most or all of the CDR can be removed and substituted by the newly incorporated sequences introduced by mutagenesis. CDRs are very accommodating to variably sized inserts without disrupting the ability of the immunoglobulin to assemble and display the newly randomized and selected amino acid residue sequence.

In one embodiment, a phage display protein can be engineered to contain multiple binding sites. For example, using the heavy chain immunoglobulin as exemplary, binding sites can be created separately by the methods of this invention into one or more of the CDRs, designated CDR1, CDR2 and CDR3. Additionally, one can introduce binding sites into a heavy chain CDR and a light chain CDR, into multiple heavy and light chain CDRs, and the like combinations.

In another embodiment, the phage display protein is engineered to include stabilization features in addition to the stabilization provided by the native structure of the display protein. To that end, cysteine residues can be coded for by the oligonucleotide, such that disulfide bridges can be formed. The placement of the cysteine residues can be varied, such that a loop structure of from about 5 to 20 amino acid residues is formed.

A preferred phagemid display protein utilizes an filamentous phage anchor fused to an immunoglobulin

heavy chain variable domain polypeptide, and the light chain associates (assembles) with the heavy chain during expression to form the displayed heterodimeric receptor, as described further herein.

5

4. Oligonucleotides

The preparation of a heterodimeric immunoglobulin molecule according to the present invention involves the use of synthetic
10 oligonucleotides designed to introduce random mutations into a preselected CDR regions of the variable domain of the heavy or light chain. Furthermore, the oligonucleotide strategy described herein has particular advantages in creating in a
15 single reaction an extremely large population of different randomized binding sites by the use of degenerate oligonucleotides.

The mutagenizing oligonucleotide randomizes the gene coding the amino acid residue sequence of the
20 immunoglobulin CDR, and the subsequent screening of the expressed phagemid display protein for preselected binding specificities is conducted as described herein and further in the Examples.

Several oligonucleotide designs were utilized to
25 form a binding site of varying lengths comprising a CDR. To that end, a series of 4, 5, 6, 8, 10 or 16 consecutive amino acid residues were randomized in the CDR region of the immunoglobulin variable domain by a degenerate oligonucleotide.

30 The general structure of an oligonucleotide for use in the present methods has the general formula ANB, where A and B define regions of homology to regions of the immunoglobulin polypeptide gene which flank the CDR region in which mutagenesis is to be
35 introduced and N defines the region of degeneracy in

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which variable amino acid residues are introduced by presenting all possible combinations of nucleotide triplets using the four bases A, T, G and C.

The number of nucleotides for each region (A, B, or N) can vary widely, but N must be in triplets so as to preserve the reading frame of the display protein. Typically, regions A and B are of sufficient length to confer hybridization specificity with the template during the primer extension reaction. Thus, regions A and B are typically each at least 6 nucleotides, and preferably each at least 9 nucleotides in length, although they can be up to about 50 nucleotides in length. The N's are typically of a widely variable length coding typically from 3 to 24 amino acid residues in length.

Where the display protein is an immunoglobulin, the homologies in regions A and B are directed to the immunoglobulin framework regions (FR) that flank the CDR into which the binding site is to be inserted.

Thus, in one embodiment, the invention contemplates an oligonucleotide useful as a primer for inducing mutagenesis in a CDR of an immunoglobulin heavy or light chain gene. The oligonucleotide has 5' and 3' termini and comprises:

i) a nucleotide sequence of about 6 to 50 nucleotides in length at the 3' termini capable of hybridizing to a first framework region of the immunoglobulin gene;

ii) a nucleotide sequence of about 6 to 50 nucleotides in length at the 5' termini capable of hybridizing to a second framework region of the immunoglobulin gene; and

iii) a nucleotide sequence between said 5' and 3' termini according to the formula:

$[NNK]_n$ or $[MNN]_n$

where n is a whole integer from 3 to 24, N is independently any nucleotide, K is G or T, M is A or C, and wherein said 5' and 3' terminal nucleotide sequences have a length of about 6 to 50 nucleotides in length, or an oligonucleotide having a sequence complementary thereto. Preferably, n is 4, 5, 6, 8, 10 or 16.

The choice of framework regions depends on the CDR into which the binding site is to be inserted. Thus, for example, for an insertion into CDR3, the 3' and 5' regions of the oligonucleotides are selected as to be complementary in nucleotide sequence to the coding strand defining FR4 and FR3 that flank CDR3, respectively, where the oligonucleotide is to be complementary to the noncoding (anti-sense) strand of the template DNA.

Furthermore, the framework region sequence varies depending upon whether an immunoglobulin heavy or light chain CDR region is being mutagenized by the present methods.

A preferred and exemplary CDR for insertion of a binding site is the CDR3 of immunoglobulin heavy or light chain. Exemplary immunoglobulin heavy and light chain polypeptides are expressed by the phagemid vector pC3AP313, described herein.

Preferred are human immunoglobulin heterodimeric molecules, and therefore, in preferred embodiments, the immunoglobulin to be mutagenized, and the oligonucleotide complementary thereto, is of human derivation.

Oligonucleotides used in the present methods that are particularly preferred for producing mutagenized heavy or light chain CDR's are described in the Examples.

As described herein, the strategy for mutagenesis

by polymerase chain reaction amplification can vary widely. Two different strategies are described in detail, differing in the oligonucleotide which introduces the degenerate nucleotides. Thus, degenerate PCR primers can be designed to be coding or non-coding depending upon whether they are the upstream or downstream PCR primer. A primer can also be designed to be complementary to those described herein and be functionally equivalent.

Similarly, the framework sequences can vary in length while maintaining the degree of mutation to the CDR, as described in the example of oligonucleotide primer pools KV6R and k10, described herein. Thus, an oligonucleotide can be comprised of varying 5' and 3' termini, and a varying amount of degenerate triplet nucleotides as described herein.

Preferred oligonucleotides for mutagenizing light chain are described in the Examples, and include the oligonucleotide primer pools KV4R, k8, KV5R, k9, KV6R, k10, KV10R, p313K38OVb, p313K310OVb and p313K316OVb. Other oligonucleotides can be utilized as is appreciated by one skilled in the art.

Oligonucleotides for use in the present invention can be synthesized by a variety of chemistries as is well known. An excellent review is "Oligonucleotide Synthesis: A Practical Approach", ed. M.J. Gait, JRL Press, New York, NY (1990). Suitable synthetic methods include, for example, the phosphotriester or phosphodiester methods see Narang et al., Meth. Enzymol., 68:90, (1979); U.S. Patent No. 4,356,270; and Brown et al., Meth. Enzymol., 68:109, (1979). Purification of synthesized oligonucleotides for use in primer extension and PCR reactions is well known. See, example Ausubel et al., "Current Protocols in Molecular Biology", John Wiley & Sons, New York,

(1987). Oligonucleotides for use in the present invention are commercially synthesized by Operon Technologies, Alameda, CA.

5. Primer Extension Reactions

The terms "polynucleotide" and "oligonucleotide" as used herein in reference to primers, probes and nucleic acid fragments or segments to be synthesized by primer extension is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depends on the ultimate conditions of use.

The term "primer" as used herein refers to a polynucleotide whether purified from a nucleic acid restriction digestion reaction or produced synthetically, which is capable of acting as a point of initiation of nucleic acid synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase, reverse transcriptase and the like, and at a suitable temperature and Ph. The primer is preferably single stranded for maximum efficiency, but may alternatively be in double stranded form. If double stranded, the primer is first treated to separate it from its complementary strand before being used to prepare extension products. Preferably, the primer is a polydeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agents for polymerization. The exact lengths of the primers will depend on many factors, including temperature and the source of primer. For example, depending on the

complexity of the target sequence, a polynucleotide primer typically contains 15 to 25 or more nucleotides, although it can contain fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template.

The primers used herein are selected to be "substantially" complementary to the different strands of each specific sequence to be synthesized or amplified. This means that the primer must be sufficiently complementary to non-randomly hybridize with its respective template strand. Therefore, the primer sequence may or may not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment can be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Such non-complementary fragments typically code for an endonuclease restriction site. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided the primer sequence has sufficient complementarity with the sequence of the strand to be synthesized or amplified to non-randomly hybridize therewith and thereby form an extension product under polynucleotide synthesizing conditions.

Primers of the present invention may also contain a DNA-dependent RNA polymerase promoter sequence or its complement. See for example, Krieg et al., Nucl. Acids Res., 12:7057-70 (1984); Studier et al., J. Mol. Biol., 189:113-130 (1986); and Molecular Cloning: A Laboratory Manual, Second Edition, Sambrook et al., eds., Cold Spring Harbor, NY (1989).

When a primer containing a DNA-dependent RNA polymerase promoter is used the primer is hybridized

to the polynucleotide strand to be amplified and the second polynucleotide strand of the DNA-dependent RNA polymerase promoter is completed using an inducing agent such as E. coli DNA polymerase I, or the Klenow fragment of E. coli DNA polymerase. The starting polynucleotide is amplified by alternating between the production of an RNA polynucleotide and DNA polynucleotide.

Primers may also contain a template sequence or replication initiation site for a RNA-directed RNA polymerase. Typical RNA-directed RNA polymerase include the QB replicase described by Lizardi et al., Biotechnology, 6:1197-1202 (1988). RNA-directed polymerases produce large numbers of RNA strands from a small number of template RNA strands that contain a template sequence or replication initiation site. These polymerases typically give a one million-fold amplification of the template strand as has been described by Kramer et al., J. Mol. Biol., 89:719-736 (1974).

The choice of a primer's nucleotide sequence depends on factors such as the distance on the nucleic acid from the region of the display protein gene into which a binding site is being introduced, its hybridization site on the nucleic acid relative to any second primer to be used, and the like.

The PCR reaction is performed using any suitable method. Generally it occurs in a buffered aqueous solution, i.e., a PCR buffer, preferably at a Ph of 7-9, most preferably about 8. Preferably, a molar excess of the primer is admixed to the buffer containing the template strand. A large molar excess of about 10^4 :1 of primer to template is preferred to improve the efficiency of the process.

The PCR buffer also contains the

deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP and a polymerase, typically thermostable, all in adequate amounts for primer extension

(polynucleotide synthesis) reaction. The resulting solution (PCR admixture) is heated to about 90 degrees Celsius (90C) - 100C for about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to 54C, which is preferable for primer hybridization. The synthesis reaction may occur at from room temperature up to a temperature above which the polymerase (inducing agent) no longer functions efficiently. Thus, for example, if DNA polymerase is used as inducing agent, the temperature is generally no greater than about 40C. An exemplary PCR buffer comprises the following: 50 Mm KCl; 10 Mm Tris-Hcl; Ph 8.3; 1.5 Mm MgCl₂; 0.001% (wt/vol) gelatin, 200 micromolar (uM) DATP; 200 uM DTTP; 200 uM DCTP; 200 uM DGTP; and 2.5 units Thermus aquaticus DNA polymerase I (U.S. Patent No. 4,889,818) per 100 microliters of buffer. Exemplary PCR amplifications are performed using the buffer system as described in the Examples.

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction

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along the template strand, until synthesis terminates, producing molecules of different lengths. There may be inducing agents, however, which initiate synthesis at the 5' end and proceed in the above direction, using the same process as described herein.

The inducing agent also may be a compound or system which will function to accomplish the synthesis of RNA primer extension products, including enzymes. In preferred embodiments, the inducing agent may be a DNA-dependent RNA polymerase such as T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase. These polymerases produce a complementary RNA polynucleotide. The high turn over rate of the RNA polymerase amplifies the starting polynucleotide as has been described by Chamberlin et al., The Enzymes, ed. P. Boyer, PP. 87-108, Academic Press, New York (1982). Another advantage of T7 RNA polymerase is that mutations can be introduced into the polynucleotide synthesis by replacing a portion of CDNA with one or more mutagenic oligodeoxynucleotides (polynucleotides) and transcribing the partially-mismatched template directly as has been previously described by Joyce et al., Nuc. Acids Res., 17:711-722 (1989). Amplification systems based on transcription have been described by Gingeras et al., in PCR Protocols, A Guide to Methods and Applications, pp 245-252, Academic Press, Inc., San Diego, CA (1990).

If the inducing agent is a DNA-dependent RNA polymerase and therefore incorporates ribonucleotide triphosphates, sufficient amounts of ATP, CTP, GTP and UTP are admixed to the primer extension reaction admixture and the resulting solution is treated as described above.

The newly synthesized strand and its

complementary nucleic acid strand form a double-stranded molecule which can be used in the succeeding steps of the process, as is known for PCR.

5 PCR is typically carried out by thermocycling i.e., repeatedly increasing and decreasing the temperature of a PCR reaction admixture within a temperature range whose lower limit is about 10C to about 40C and whose upper limit is about 90C to about 100C. The increasing and decreasing can be
10 continuous, but is preferably phasic with time periods of relative temperature stability at each of temperatures favoring polynucleotide synthesis, denaturation and hybridization.

15 PCR amplification methods are described in detail in U.S. Patent Nos. 4,683,192, 4,683,202, 4,800,159, and 4,965,188, and at least in several texts including "PCR Technology: Principles and Applications for DNA Amplification", H. Erlich, ed., Stockton Press, New York (1989); and "PCR Protocols: A Guide to Methods
20 and Applications", Innis et al., eds., Academic Press, San Diego, California (1990); the teachings of which are hereby incorporated by reference.

25 PCR can be conducted to ligate two different PCR reaction products in a method referred to as overlapping PCR or crossover PCR. This method is used to connect heavy and light chain PCR reaction products, and is described herein. In the overlapping PCR method, it is convenient to introduce the
30 mutagenesis of a CDR by designing either the 3' primer or the 5' primer as the degenerate oligonucleotide in the primer pair. Both methods are described in the Examples.

35 Additional preferred PCR reactions using the oligonucleotides and methods of this invention are described in the Examples.

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6. Phage Display Vectors

Random mutagenesis of CDRs in a variable (V) region and screening methods such as is described by Barbas et al, Proc. Natl. Acad. Sci., USA, 89:4457-4461, (1992) are used for preparing antibody libraries that contain diverse binding site specificities with the improvements described herein.

The methods of the present invention for preparing antibody molecules involve the use of phage display vectors for their particular advantage of providing a means to screen a very large population of expressed display proteins and thereby locate one or more specific clones that code for a desired binding reactivity.

The use of phage display vectors derives from the previously described use of combinatorial libraries of antibody molecules based on phagemids. The combinatorial library production and manipulation methods have been extensively described in the literature, and will not be reviewed in detail herein, except for those features required to make and use unique embodiments of the present invention. However, the methods generally involve the use of a filamentous phage (phagemid) surface expression vector system for cloning and expressing antibody species of the library.

Various phagemid cloning systems for producing combinatorial libraries have been described by others. See for example the preparation of combinatorial antibody libraries on phagemids as described by Kang et al., Proc. Natl. Acad. Sci., USA, 88:4363-4366 (1991); Barbas et al., Proc. Natl. Acad. Sci., USA, 88:7978-7982 (1991); Zebedee et al., Proc. Natl. Acad. Sci., USA, 89:3175-3179 (1992); Kang et al., Proc. Natl. Acad. Sci., USA, 88:11120-11123 (1991); Barbas

et al., Proc. Natl. Acad. Sci., USA, 89:4457-4461 (1992); and Gram et al., Proc. Natl. Acad. Sci., USA, 89:3576-3580 (1992), the disclosures of which are hereby incorporated by reference.

5

a. Phage Display Vector Structure

A preferred phagemid vector of the present invention is a recombinant DNA (RDNA) molecule containing a nucleotide sequence that codes for and is capable of expressing a fusion polypeptide containing, in the direction of amino- to carboxy-terminus, (1) a prokaryotic secretion signal domain, (2) a heterologous polypeptide defining an immunoglobulin heavy or light chain variable region, and (3) a filamentous phage membrane anchor domain. The vector includes DNA expression control sequences for expressing the fusion polypeptide, preferably prokaryotic control sequences.

The filamentous phage membrane anchor is preferably a domain of the cpIII or cpVIII coat protein capable of associating with the matrix of a filamentous phage particle, thereby incorporating the fusion polypeptide onto the phage surface.

Preferred membrane anchors for the vector are obtainable from filamentous phage M13, f1, fd, and equivalent filamentous phage. Preferred membrane anchor domains are found in the coat proteins encoded by gene III and gene VIII. The membrane anchor domain of a filamentous phage coat protein is a portion of the carboxy terminal region of the coat protein and includes a region of hydrophobic amino acid residues for spanning a lipid bilayer membrane, and a region of charged amino acid residues normally found at the cytoplasmic face of the membrane and extending away from the membrane.

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In the phage f1, gene VIII coat protein's membrane spanning region comprises residue Trp-26 through Lys-40, and the cytoplasmic region comprises the carboxy-terminal 11 residues from 41 to 52 (Ohkawa et al., J. Biol. Chem., 256:9951-9958, 1981). An exemplary membrane anchor would consist of residues 26 to 40 of cpviii. Thus, the amino acid residue sequence of a preferred membrane anchor domain is derived from the M13 filamentous phage gene VIII coat protein (also designated cpviii or CP 8). Gene VIII coat protein is present on a mature filamentous phage over the majority of the phage particle with typically about 2500 to 3000 copies of the coat protein.

In addition, the amino acid residue sequence of another preferred membrane anchor domain is derived from the M13 filamentous phage gene III coat protein (also designated cpiii). Gene III coat protein is present on a mature filamentous phage at one end of the phage particle with typically about 4 to 6 copies of the coat protein.

For detailed descriptions of the structure of filamentous phage particles, their coat proteins and particle assembly, see the reviews by Rached et al., Microbiol. Rev., 50:401-427 (1986); and Model et al., in "The Bacteriophages: Vol. 2", R. Calendar, ed. Plenum Publishing Co., pp. 375-456 (1988).

The secretion signal is a leader peptide domain of a protein that targets the protein to the periplasmic membrane of gram negative bacteria. A preferred secretion signal is a pelB secretion signal. The predicted amino acid residue sequences of the secretion signal domain from two pelB gene product variants from Erwinia carotova are described in Lei et al., Nature, 331:543-546 (1988).

The leader sequence of the pelB protein has

previously been used as a secretion signal for fusion proteins (Better et al., Science, 240:1041-1043 (1988); Sastry et al., Proc. Natl. Acad. Sci., USA, 86:5728-5732 (1989); and Mullinax et al., Proc. Natl. Acad. Sci., USA, 87:8095-8099 (1990)). Amino acid residue sequences for other secretion signal polypeptide domains from E. coli useful in this invention as described in Oliver, Escherichia coli and Salmonella Typhimurium, Neidhard, F.C. (ed.), American Society for Microbiology, Washington, D.C., 1:56-69 (1987).

DNA expression control sequences comprise a set of DNA expression signals for expressing a structural gene product and include both 5' and 3' elements, as is well known, operatively linked to the cistron such that the cistron is able to express a structural gene product. The 5' control sequences define a promoter for initiating transcription and a ribosome binding site operatively linked at the 5' terminus of the upstream translatable DNA sequence.

The 3' control sequences define at least one termination (stop) codon in frame with and operatively linked to the heterologous fusion polypeptide.

In preferred embodiments, the vector used in this invention includes a prokaryotic origin of replication or replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extra-chromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such origins of replication are well known in the art. Preferred origins of replication are those that are efficient in the host organism. A preferred host cell is E. coli. A preferred strain of E. coli is the supE strain as an amber stop codon is translated as glutamine (Q). For

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use of a vector in E. coli, a preferred origin of replication is ColE1 found in pBR322 and a variety of other common plasmids. Also preferred is the p15A origin of replication found on pACYC and its derivatives. The ColE1 and p15A replicon have been extensively utilized in molecular biology, are available on a variety of plasmids and are described at least by Sambrook et al., in "Molecular Cloning: a Laboratory Manual", 2nd edition, Cold Spring Harbor Laboratory Press, New York (1989).

The ColE1 and p15A replicons are particularly preferred for use in one embodiment of the present invention where two "binary" plasmids are utilized because they each have the ability to direct the replication of plasmid in E. coli while the other replicon is present in a second plasmid in the same E. coli cell. In other words, ColE1 and p15A are non-interfering replicons that allow the maintenance of two plasmids in the same host (see, for example, Sambrook et al., supra, at pages 1.3-1.4). This feature is particularly important when using binary vectors because a single host cell permissive for phage replication must support the independent and simultaneous replication of two separate vectors, for example when a first vector expresses a heavy chain polypeptide and a second vector expresses a light chain polypeptide, and the admixture of libraries of heavy and light chain gene is desired to combine all possible combinations of heavy and light chain.

In addition, those embodiments that include a prokaryotic replicon can also include a gene whose expression confers a selective advantage, such as drug resistance, to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin, tetracycline,

neomycin/kanamycin or chloramphenicol. Vectors typically also contain convenient restriction sites for insertion of translatable DNA sequences.

Exemplary vectors are the plasmids pUC8, pUC9, PBR322, and pBR329 available from BioRad Laboratories, (Richmond, CA) and pPL and pKK223 available from Pharmacia, (Piscataway, NJ).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting between different genetic environments another nucleic acid to which it has been operatively linked. Preferred vectors are those capable of autonomous replication and expression of structural gene products present in the DNA segments to which they are operatively linked. Vectors, therefore, preferably contain the replicons and selectable markers described earlier.

As used herein with regard to DNA sequences or segments, the phrase "operatively linked" means the sequences or segments have been covalently joined, preferably by conventional phosphodiester bonds, into one strand of DNA, whether in single or double stranded form, in a manner such that the sequences are able to function in the vector, ie., to be expressed. The choice of vector to which a transcription unit or a cassette of this invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules.

In a preferred embodiment, the vector is capable of co-expression of two cistrons contained therein, such as a heavy chain gene and a light chain gene. Co-expression has been accomplished in a variety of systems and therefore need not be limited to any

particular design, so long as sufficient relative amounts of the two gene products are produced to allow assembly and expression of functional heterodimer. Preferred vectors capable of co-expression are described herein.

In a preferred embodiment, a DNA expression vector is designed for convenient manipulation in the form of a filamentous phage particle encapsulating a genome according to the teachings of the present invention. In this embodiment, a DNA expression vector further contains a nucleotide sequence that defines a filamentous phage origin of replication such that the vector, upon presentation of the appropriate genetic complementation, can replicate as a filamentous phage in single stranded replicative form and be packaged into filamentous phage particles. This feature provides the ability of the DNA expression vector to be packaged into phage particles for subsequent segregation of the particle, and vector contained therein, away from other particles that comprise a population of phage particles.

A filamentous phage origin of replication is a region of the phage genome, as is well known, that defines sites for initiation of replication, termination of replication and packaging of the replicative form produced by replication (see for example, Rasched et al., Microbiol. Rev., 50:401-427, 1986; and Horiuchi, J. Mol. Biol., 188:215-223, 1986). A preferred filamentous phage origin of replication for use in the present invention is an M13, f1 or fd phage origin of replication (Short et al., Nucl. Acids Res., 16:7583-7600, 1988).

A preferred DNA expression vector for cloning, mutagenesis and expressing a phagemid display protein of this invention is the dicistronic phagemid

expression vector pC3AP313 described herein. pC3AP313 is capable of co-expressing both the phagemid display protein containing a heavy chain fusion and the light chain.

5 It is to be understood that, due to the genetic code and its attendant redundancies, numerous polynucleotide sequences can be designed that encode a contemplated heavy or light chain immunoglobulin variable region amino acid residue sequence. Thus,
10 the invention contemplates such alternate polynucleotide sequences incorporating the features of the redundancy of the genetic code, and sequences complementary thereto.

Insofar as the expression vector for producing a
15 human monoclonal antibody of this invention is carried in a host cell compatible with expression of the antibody, the invention contemplates a host cell containing a vector or polynucleotide of this invention. A preferred host cell is E. coli, as
20 described herein.

The preferred phagemid expression vector in the form of plasmid that produces a phagemid display protein of this invention was deposited pursuant to Budapest Treaty requirements with the American Type
25 Culture Collection (ATCC), Rockville, MD. The phagemid expression vector pC3AP313 has the respective ATCC Accession Number 75408, and includes a preferred immunoglobulin light chain variable domain polypeptide encoding gene.

30 b. Use of Phagemid Display Vectors to Produce Antibody Libraries

A phagemid vector for use herein is a recombinant DNA (RDNA) molecule containing a
35 nucleotide sequence that codes for and is capable of

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expressing an antibody-derived heterodimeric protein on the surface of the phagemid in the form of a phagemid display protein. An exemplary and preferred phagemid vector is the plasmid pC3AP313 described in the Examples.

The method for producing a heterodimeric immunoglobulin molecule generally involves (1) introducing a heavy or light chain V region-coding gene of interest into the phagemid display vector; (2) introducing a randomized binding site into the phagemid display protein vector by primer extension with an oligonucleotide containing regions of homology to a CDR of the antibody V region gene and containing regions of degeneracy for producing randomized coding sequences as described herein, to form a large population of display vectors each capable of expressing different putative binding sites displayed on a phagemid surface display protein, (3) expressing the display protein and binding site on the surface of a filamentous phage particle, and (3) isolating (screening) the surface-expressed phage particle using affinity techniques such as panning of phage particles against a preselected antigen, thereby isolating one or more species of phagemid containing a display protein containing a binding site that binds a preselected antigen.

As a further characterization of the produced antibody binding site, the nucleotide and corresponding amino acid residue sequence of the gene coding the randomized CDR is determined by nucleic acid sequencing. The primary amino acid residue sequence information provides essential information regarding the binding site's reactivity.

An exemplary preparation of an antibody binding site in the CDR3 of the variable domains of the heavy

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and light chains of an immunoglobulin heterodimer is described in the Examples. The isolation of a particular vector capable of expressing an antibody binding site of interest involves the introduction of the dicistronic expression vector able to express the phagemid display protein into a host cell permissive for expression of filamentous phage genes and the assembly of phage particles. Typically, the host is E. coli. Thereafter, a helper phage genome is introduced into the host cell containing the phagemid expression vector to provide the genetic complementation necessary to allow phage particles to be assembled.

The resulting host cell is cultured to allow the introduced phage genes and display protein genes to be expressed, and for phage particles to be assembled and shed from the host cell. The shed phage particles are then harvested (collected) from the host cell culture media and screened for desirable antibody binding properties. Typically, the harvested particles are "panned" for binding with a preselected antigen. The strongly binding particles are then collected, and individual species of particles are clonally isolated and further screened for binding to the antigen. Phage which produce a binding site of desired antigen binding specificity are selected.

A number of different permutations for manipulation of a phagemid display vector for practicing the present invention are described herein, but the invention need not be limited.

The invention describes, in one embodiment, a method for producing an antibody combining site in a polypeptide of either the heavy or light chain of a heterodimer that comprises inducing mutagenesis in a complementarity determining region of an

immunoglobulin heavy or light chain gene which
comprises amplifying a CDR portion of the
immunoglobulin gene by PCR using a PCR primer
oligonucleotide of this invention to introduce random
mutagenesis into the CDR portion.

7. Universal Light Chain

The present invention also describes the
discovery of immunoglobulin light chains which have
the ability to complex into a functional heterodimer
with any of a variety of heavy chains, and therefore
are referred to as universal light chains to connote
their ability to be used with a variety of heavy
chains.

Of particular utility is the ease and diversity
in producing large antibody repertoires using a
universal light chain. In one approach, a universal
light chain is crossed with a heavy chain library,
such as a randomized heavy chain. In a particular
embodiment, a heavy chain of preferred specificity is
randomized by CDR mutagenesis, and the resulting heavy
chain library is crossed with a universal light chain
to form an antibody repertoire which is then screened
for desirable binding affinities. This approach
provides optimization of a known heavy chain to
produce improved binding specificity. The use of a
universal light chain increases the number of
combinations which yield functional heterodimeric
antibody molecules.

In another embodiment, the invention contemplates
the use of universal light chain as a framework for
mutagenesis to yield a library of modified universal
light chain genes. This light chain library can be
used to optimize a known heavy chain, or can be
crossed with a heavy chain library, as described

herein.

Universal light chain is an immunoglobulin light chain polypeptide that includes at least one CDR and has the capacity to complex with a substantial variety of heavy chains in a heavy chain library. By "substantial variety of heavy chains in a heavy chain library" is meant that the universal light chain complexes with at least 0.1% of the heavy chain species in a heavy chain library, preferably with at least 1%, and more preferably with at least 10% of the heavy chain species in a heavy chain library.

A preferred universal light chain has the sequence characteristics of the light chain amino acid residue sequence shown in SEQ ID NO 2 or the sequence encoded by the light chain gene in plasmid pC3AP313 deposited with the ATCC as Accession No. ATCC 75408. A preferred universal light chain may also have the sequence characteristics of the light chain amino acid residue sequence shown in SEQ ID NO 62 or the sequence encoded by the light chain gene in plasmid p6F described in Example 8B1. By sequence characteristics is meant that the expressed light chain protein functions in a similar manner as the light chains shown in SEQ ID NOs 2 and 62. Similarity is indicated where the expressed light chain gene functionally associates with the same, or substantially the same, heavy chain genes to produce a heterodimer which immunocomplexes antigen with the same or substantially same immunoaffinity as a heterodimer formed with the light chains shown in SEQ ID NOs 2 and 62. Preferably, a universal light chain includes an amino acid residue sequence shown in SEQ ID NOs 2 and 62.

Thus, in one embodiment, the invention contemplates the preparation of a heterodimeric immunoglobulin (antibody) molecule having variable

domain heavy and light chain polypeptides using a universal light chain gene in a cross with a library of heavy chain genes, followed by expression and screening according to the present invention. The method comprises the steps of:

a) combining an immunoglobulin variable domain light chain gene that includes a sequence having the sequence characteristics of the light chain shown in SEQ ID NO 2 or 62 with one or more immunoglobulin variable domain heavy chain genes to form a combinatorial immunoglobulin heavy and light chain gene library, said combining comprising operatively linking said light chain gene with one of said heavy chain genes in a vector capable of co-expression of said heavy and light chain genes;

b) expressing the combinatorial gene library to form a combinatorial antibody library of expressed heavy and light chain polypeptides; and

c) selecting species of said combinatorial antibody library for the ability to bind a preselected antigen.

In preferred embodiments, the heavy chain library used in the foregoing method is a randomized heavy chain library with a mutagenized CDR domain. In preferred embodiments, the immunoglobulin light chain gene used in the foregoing method has the sequence characteristics of the light chain gene in SEQ ID NO 2 or 62.

In another embodiment, the invention contemplates the use of universal light chain in the mutagenesis methods to form a light chain library according to the present invention. Mutagenesis of light chain in this manner can be conducted in a variety of ways, such as is described in detail in the Examples.

Examples

The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention.

Moreover, such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

1. Production of Phagemid-Displayed Fab Heavy and Light Chain Heterodimers that Bind to Synthetic Hapten Conjugates

In practicing this invention to obtain expression of Fab antibodies having anti-hapten binding sites, the Fabs of which are expressed on a phage surface, the heavy (Fd consisting of V_H and C_H1) and light (κ) chains (V_L , C_L) of antibodies were first targeted to the periplasm of E. coli for the assembly of heterodimeric Fab molecules. In this system, the first cistron encoded a periplasmic secretion signal (pelB leader) operatively linked to the fusion protein, Fd-cpIII. The second cistron encoded a second pelB leader operatively linked to a kappa light chain. The presence of the pelB leader facilitated the coordinated but separate secretion of both the fusion protein containing the native as well as semisynthetic binding site and light chain from the bacterial cytoplasm into the periplasmic space.

In this process, each chain was delivered to the periplasmic space by the pelB leader sequence, which was subsequently cleaved. The heavy chain was anchored in the membrane by the cpIII membrane anchor domain while the light chain was secreted into the

periplasm. Fab molecules were formed from the binding of the heavy chain with the soluble light chains. In addition, the expression vectors used in this invention allow for the production of soluble Fab heterodimers as described in Example 5C.

A. Preparation of a Dicistronic Expression Vector, pComb3, Capable of Expressing a Phagemid Fab Display Protein

The Pcomb3 phagemid expression vector of this invention is used in expressing the anti-hapten antibodies. The antibody Fd chain comprising variable (V_H) and constant (C_H1) domains of the heavy chain were fused with the C-terminal domain of bacteriophage gene III (3) coat protein. Gene III of filamentous phage encodes a 406-residue minor phage coat protein, cpiii (cp3), which is expressed prior to extrusion in the phage assembly process on a bacterial membrane and accumulates on the inner membrane facing into the periplasm of E. coli.

The phagemid vector, designated Pcomb3, allowed for both surface display and soluble forms of Fabs. The vector was originally designed for the cloning of combinatorial Fab libraries as described by Barbas et al., Methods, A Companion to Methods in Enzymology, 2:119-124 (1991), the disclosure of which is hereby incorporated by reference.

The Xho I and Spe I sites were provided for cloning complete PCR-amplified heavy chain (Fd) sequences. An Aat II restriction site is also present that allows for the insertion of Xho I/Aat II digests of the PCR products. The Sac I and Xba I sites were provided for cloning PCR amplified antibody light chains of this invention. The cloning sites were compatible with previously reported mouse and human

PCR primers as described by Huse et al., Science,
246:1275-1281 (1989) and Persson et al., Proc. Natl.
Acad. Sci., USA, 88:2432-2436 (1991). The nucleotide
sequence of the pelB, a leader sequence for directing
the expressed protein to the periplasmic space, was as
reported by Huse et al., supra.

The vector also contained a ribosome binding site
as described by Shine et al., Nature, 254:34 (1975).
The sequence of the phagemid vector, pBluescript,
which includes ColE1 and F1 origins and a
beta-lactamase gene, has been previously described by
Short et al., Nuc. Acids Res., 16:7583-7600 (1988) and
has the GenBank Accession Number 52330 for the
complete sequence. Additional restriction sites, Sal
I, Acc I, Hinc II, Cla I, Hind III, Eco RV, Pst I and
Sma I, located between the Xho I and Spe I sites of
the empty vector were derived from a 51 base pair
stuffer fragment of Pbluescript as described by Short
et al., supra. A nucleotide sequence that encodes a
flexible 5 amino acid residue tether sequence which
lacks an ordered secondary structure was juxtaposed
between the Fab and cp3 nucleotide domains so that
interaction in the expressed fusion protein was
minimized.

Thus, the resultant combinatorial vector, Pcomb3,
consisted of a DNA molecule having two cassettes to
express one fusion protein, Fd/cp3, and one soluble
protein, the light chain. The vector also contained
nucleotide residue sequences for the following
operatively linked elements listed in a 5' to 3'
direction: a first cassette consisting of LacZ
promoter/operator sequences; a Not I restriction site;
a ribosome binding site; a pelB leader; a spacer
region; a cloning region bordered by 5' Xho and 3' Spe
I restriction sites; the tether sequence; the

sequences encoding bacteriophage cp3 followed by a stop codon; a Nhe I restriction site located between the two cassettes; a second lacZ promoter/operator sequence followed by an expression control ribosome binding site; a pelB leader; a spacer region; a cloning region bordered by 5' Sac I and a 3' Xba I restriction sites followed by expression control stop sequences and a second Not I restriction site.

In the above expression vector, the Fd/cp3 fusion and light chain proteins were placed under the control of separate lac promoter/operator sequences and directed to the periplasmic space by pelB leader sequences for functional assembly on the membrane. Inclusion of the phage F1 intergenic region in the vector allowed for the packaging of single-stranded phagemid with the aid of helper phage. The use of helper phage superinfection allowed for the expression of two forms of cp3. Consequently, normal phage morphogenesis was perturbed by competition between the Fd/cp3 fusion and the native cp3 of the helper phage for incorporation into the virion. The resulting packaged phagemid carried native cp3, which is necessary for infection, and the encoded Fab fusion protein, which is displayed for selection. Fusion with the C-terminal domain was necessitated by the phagemid approach because fusion with the infective N-terminal domain would render the host cell resistant to infection.

The Pcomb3 expression vector described above forms the basic construct of the Fab display phagemid expression vectors described below used in this invention for the production of human anti-hapten Fab antibodies. The surface display phagemid expression vector, pC3AP313, was deposited with ATCC on February 2, 1993 for use in this invention. The deposited

vector has been assigned the ATCC Accession Number 75408. The pC3AP313 expression vector contained the bacteriophage gene III and heavy and light chain variable domain sequences for encoding human Fab antibodies against tetanus toxoid. The coding DNA strand nucleotide sequences of the anti-tetanus toxoid heavy and light chain variable domains in pC3AP313 are respectively listed in the Sequence Listing under SEQ ID NO 1 and 2. The reading frame of the nucleotide sequences for translation into amino acid residue sequences begins at nucleotide position 1 for both the light and heavy chain variable domains of pC3AP313. The tetanus toxoid-specific sequences were originally obtained from screening phage lambda vector combinatorial libraries of antibody heavy and light chains derived from the peripheral blood lymphocytes of an individual immunized with tetanus toxoid as described by Persson et al., supra, the disclosure of which is hereby incorporated by reference. Clone 3 was selected from the library screening and the heavy and light chain sequences were then respectively isolated by restriction digestion with Xho I/Spe I and Sac I/Xba I and ligated into a similarly digested Pcomb3 vector. The ligation procedure in creating expression vector libraries and the subsequent expression of the anti-hapten Fab antibodies is performed as described in Example 2.

2. Selection of Human Anti-Hapten Antibodies from Semisynthetic Light and Heavy Chain Libraries

A. Preparation of Randomized Sites Within the Light Chain CDR3 of a Phagemid Fab Display Protein Produced by a Dicistronic Expression Vector

1) PCR with Coding Degenerate
Oligonucleotide Primers

Semisynthetic human Fab libraries in which both the CDR3 heavy and light chain domains were randomized were constructed, displayed on the surface of filamentous phage and selected for binding to three hapten conjugates. The phagemid expression vector, pC3AP313, containing heavy and light chain sequences for encoding a human antibody that immunoreacted with tetanus toxin, was used as a template for PCR.

Light chain libraries having CDR3 randomized in predetermined amino acid residue positions were prepared using the overlap PCR amplification protocols described herein. In the libraries, oligonucleotide primer pools were designed to result in the formation of CDR3 in lengths of 8, 9 and 10 amino acids to correspond to the naturally occurring loop lengths in humans. Diversity was limited to Kabat positions 92-96 as the remaining four positions are highly conserved in nature.

To amplify the 5' end of the light chain from framework 1 to the end of framework 3 of pC3AP313, the following primer pairs were used. The 5' coding (sense) oligonucleotide primer, KEF, having the nucleotide sequence 5'GAATTCTAAACTAGCTAGTCG3' (SEQ ID NO 3), hybridized to the noncoding strand of the light chain corresponding to the region 5' of and including the beginning of framework 1. The 3' noncoding (antisense) oligonucleotide primer, KV12B, having the nucleotide sequence 5'ATACTGCTGACAGTAATACAC3' (SEQ ID NO 4), hybridized to the coding strand of the light chain corresponding to the 3' end of the framework 3 region. The oligonucleotide primers were synthesized by Operon Technologies, Alameda, CA. The terms coding

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or sense, used in the context of oligonucleotide primers, identifies a primer that is the same sequence as the DNA strand that encodes a heavy or light chain and that hybridizes to the noncoding strand.

5 Similarly, the term noncoding or antisense identifies a primer that is complementary to the coding strand and thus hybridizes to it.

For overlap PCR, each set of PCR reactions were performed in a 100 microliter (ul) reaction containing
10 1 microgram (ug) of each of oligonucleotide primers listed above in a particular pairing, 8 ul 2.5 Mm dNTP's (DATP, DCTP, DGTP, DTTP), 1 ul Taq polymerase, 10 ng of template pC3AP313, and 10 ul of 10X PCR buffer purchased commercially (Promega Biotech,
15 Madison, WI). Thirty-five rounds of PCR amplification in a Perkin-Elmer Cetus 9600 GeneAmp PCR System thermocycler were then performed. The amplification cycle consisted of denaturing at 94 degrees C (94C) for 1 minute, annealing at 47C for 1 minute, followed
20 by extension at 72C for 2 minutes. To obtain sufficient quantities of amplification product, 15 identical PCR reactions were performed.

The resultant PCR amplification products were then gel purified on a 1.5% agarose gel using standard
25 electroelution techniques as described in "Molecular Cloning: A Laboratory Manual", Sambrook et al., eds., Cold Spring Harbor, NY (1989). Briefly, after gel electrophoresis, the region of the gel containing the DNA fragments of predetermined size was excised,
30 electroeluted into a dialysis membrane, ethanol precipitated and resuspended in buffer containing 10 millimolar (Mm) Tris-Hcl [Tris(hydroxymethyl)aminomethane-hydrochloride] at Ph 7.5 and 1 Mm EDTA (ethylenediaminetetraacetic acid) to
35 a final concentration of 50 nanograms/milliliter

(ng/ml).

The purified amplification products were then used in an overlap extension PCR reaction with the products of the second PCR reaction, both as described below, to recombine the two products into reconstructed variable domain light chains containing the mutagenized third domain of the complementarity determining region (CDR3).

The second PCR reaction resulted in the amplification of the light chain from the 3' end of framework region 3 extending to the end of light chain constant region. To amplify this region for encoding a 4 random amino acid residue sequence in the CDR3 having a total length of 8 amino acids, the following primer pairs were used. The 5' coding oligonucleotide primer pool, designated KV4R, had the nucleotide sequence represented by the formula,
5'TATTACTGTCAGCAGTATNNKNNKNNKNNKACTTTCGGCGGAGGGACCAAGG
TGGAG3' (SEQ ID NO 5), where N can be A, C, G, or T and K is either G or T. The 3' noncoding primer, T7B, hybridized to the coding strand at the 3' end of the light chain constant domain having the sequence
5'AATACGACTCACTATAGGGCG3' (SEQ ID NO 6). The 5' end of the primer pool is complementary to the 3' end of framework 3 represented by the complementary nucleotide sequence of the oligonucleotide primer KV12B and the 3' end of the primer pool is complementary to the 5' end of framework 4. The region between the two specified ends of the primer pool is represented by a 12-mer NNK degeneracy. The second PCR reaction was performed on the pC3AP313 vector in a 100 ul reaction as described above containing 1 ug of each of oligonucleotide primers. The resultant PCR products encoded a diverse population of 4 mutagenized amino acid residues in a

light chain CDR3 having a total of 8 amino acid residues. In the resultant CDR3, the 4 mutagenized amino acid residue positions were bordered on the amino terminal side by 3 amino acid residues that were left unchanged, Gln-Gln-Tyr, and on the carboxy terminal side by one amino acid residue, Thr. The products were then gel purified as described above.

An alternative oligonucleotide pool for preparing 4 randomized amino acid residues in a CDR3 having 8 amino acid residues was designated k8 having the formula

5' TATTACTGTCAGCAGTATNNKNNKNNKNNKACTTTCGGCGGAGGGACC3' (SEQ ID NO 7). The k8 primer lacked 9 nucleotides from the 3' end of KV4R.

One hundred nanograms of gel purified products from the first and second PCR reactions were then admixed with 1 ug each of KEF and T7B oligonucleotide primers as a primer pair in a final PCR reaction to form a complete light chain fragment by overlap extension. The PCR reaction admixture also contained 10 ul of 10X PCR buffer, 1 ul Taq polymerase and 8 ul 2.5 Mm DNTP'S as described above.

To obtain sufficient quantities of amplification product, 15 identical overlap PCR amplifications were performed. The resulting light chain fragments beginning at framework 1 and extending to the end of constant region of the light chain thus contained a randomly mutagenized CDR3 region for encoding 4 new amino acid residues. The light chain fragment amplification products from the 15 reactions were first pooled and then gel purified as described above prior to their incorporation into the pC3AP313 surface display phagemid expression vector to form a library as described in Example 4A. The light chain library having a CDR3 of 8 amino acids resulting from

amplifications with either KV4R or k8 was designated K8.

To create a randomized light chain CDR3 for encoding a CDR3 having a total of 9 amino acids in which 5 amino acid residues were randomized, the KV5R primer was used with the 3' primer, T7B, previously described. The KV5R had the formula
5' TATTACTGTCAGCAGTATNNKNNKNNKNNKNNKACTTTCGGCGGAGGGACCA
AGGTGGAG3' (SEQ ID NO 8), where N is A, C, G or T and K is G or T.

An alternative oligonucleotide pool for preparing 5 randomized amino acid residues in a CDR3 having 9 amino acid residues was designated k9 having the formula
5' TATTACTGTCAGCAGTATNNKNNKNNKNNKNNKACTTTCGGCGGAGGGACC3
' (SEQ ID NO 9), where N is A, C, G or T and K is G or T. The k9 primer lacked 9 nucleotides from the 3' end of KV5R.

The resultant PCR products from amplifications with either KV5R or k9 encoded a diverse population of 5 mutagenized amino acid residues in a light chain CDR3 having a total of 9 amino acid residues. In the resultant CDR3, the 5 mutagenized amino acid residue positions were bordered on the amino terminal side by 3 amino acid residues that were left unchanged, Gln-Gln-Tyr, and on the carboxy terminal side by one amino acid residue, Thr. The light chain library having a CDR3 of 9 amino acids resulting from this amplification was designated K9.

To create a randomized light chain CDR3 for encoding a CDR3 having a total of 10 amino acids in which 6 amino acid residues were randomized, the KV6R primer was used with the 3' primer, T7B, previously described. The KV6R primer had the formula
5' GATTTTGCAGTGTATTACTGTCAGCAGTATNNKNNKNNKNNKNNKNNKACTT

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TCGGCGGAGGGACCAAGGTGGAG3' (SEQ ID NO 10), where N is A, C, G or T and K is G or T.

An alternative oligonucleotide pool for preparing 6 randomized amino acid residues in a CDR3 having 10 amino acid residues was designated k10 having the formula

5' TATTACTGTCAGCAGTATNNKNNKNNKNNKNNKNNKNNKACTTTTCGGCGGAGGGA
CC3', where N is A, C, G or T and K is G or T (SEQ ID
NO 11). The k10 primer was shortened on both the 5'
and 3' ends of the KV6R primer by 12 and 9
nucleotides, respectively.

The resultant PCR products from amplifications with either KV6R or k10 encoded a diverse population of 6 mutagenized amino acid residues in a light chain CDR3 having a total of 10 amino acid residues. The light chain library having a CDR3 of 10 amino acids resulting from this amplification was designated K10. In the resultant CDR3, the 6 mutagenized amino acid residue positions were bordered on the amino terminal side by 3 amino acid residues that were left unchanged, Gln-Gln-Tyr, and on the carboxy terminal side by one amino acid residue, Thr.

To create a randomized light chain CDR3 for encoding a CDR3 having a total of 10 amino acids in which all 10 amino acid residues were randomized, the KV10R primer was used with the 3' primer, T7B, previously described. The KV10R primer had the formula

5' GATTTTGCAGTGTATTACTGTNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKT
TCGGCGGAGGGACCAAGGTGGAG3' (SEQ ID NO 12), where N is
A, C, G or T and K is G or T.

The resultant PCR products encoded a diverse population of 10 mutagenized amino acid residues in a light chain CDR3 having a total of 10 amino acid residues. The light chain library having a CDR3 of 10

amino acids resulting from this amplification was designated K10'.

2) PCR with Noncoding Degenerate
Oligonucleotide Primers

Additional semisynthetic human Fab libraries in which both the heavy and light chain CDR3 were randomized were constructed, displayed on the surface of filamentous phage and selected for binding to three hapten conjugates. Another way of introducing randomized nucleotides into a template DNA sequence for encoding amino acid residue substitutions or additions was to use noncoding degenerate primers instead of using coding degenerate oligonucleotide primers as described above in Example 2A1). The coding (sense) degeneracy had the formula 5'-NNK-3', where N can be either A, C, G or T and K is either G or T. For use in this invention, the noncoding (antisense) oligonucleotide primers used in overlap PCR procedures had the degeneracy formula 5'-MNN-3' written in the conventional 5' to 3' direction, where M is equal to either A or C. Written in 3' to 5' direction, the noncoding oligonucleotide had the formula 3'-NNM-5' which is that complementary sequence to the coding formula 5'-NNK-3'. Thus, the noncoding oligonucleotide primers used in this invention provided for incorporating the same coding sequence degeneracies as the coding oligonucleotide primers. In other words, the same semisynthetic library having a particular CDR randomized arrangement can be obtained by using overlap PCR with predetermined coding or noncoding primers. The use of a noncoding primer also requires the use of different overlap primers as described herein.

The resultant PCR products were also prepared

from the phagemid expression vector, pC3AP313, containing heavy and light chain sequences for encoding a human antibody that immunoreacted with tetanus toxin.

5 Light chain libraries having CDR3 randomized in predetermined amino acid residue positions were prepared using the overlap PCR amplification protocols described herein. In the libraries, oligonucleotide primer pools were designed to result in the formation
10 of CDR3 in lengths of 8, 10 and 16 amino acids in length. For all three libraries, the CDR3 was completely randomized using the noncoding degeneracy 5'-MNN-3' that was complementary to the coding degeneracy 5'-NNK-3' as used in primers described in
15 Example 2A1).

 To amplify the 5' end of the light chain from framework 1 to the end of CDR3 of pC3AP313 and to incorporate degenerate nucleotide sequences into the amplified DNA, the following primer pairs were used.
20 The 5' coding (sense) oligonucleotide primer, KEF, having the nucleotide sequence 5'GAATTCTAAACTAGCTAGTCG3' (SEQ ID NO 3), hybridized to the noncoding strand of the light chain corresponding to the region 5' of and including the beginning of
25 framework 1. Three separate noncoding (antisense) oligonucleotide primer pools were designed to prepare light chain CDR3 libraries having 8, 10 or 16 randomized amino acid residues. The degenerate oligonucleotides overlapped with the 3' end of
30 framework region 3 through the CDR3 into the 5' end of framework region 4.

 The primer pool designated p313K380Vb for incorporating 8 randomized amino acid residues had the noncoding nucleotide sequence written in the 5' to 3'
35 direction,

5' GTTCCACCTTGGTCCCTTGGCCGAAMNNNMNNNMNNNMNNNMNNNMNNACAGTAGTACACTGCAAAATC3', where M is either A or C, and N can be A, C, G or T (SEQ ID NO 13). The light chain library formed from this amplification was designated CDR3-LCNC8. The primer pool, designated p313K310OVb, for incorporating 10 randomized amino acid residues had the noncoding nucleotide sequence written in the 5' to 3' direction,

5' GTTCCACCTTGGTCCCTTGCGCCGAAMNNMNNNMNNMNMMNMMNNMMNNMNN
MNNACAGTAGTACTGCAAAATC3', where M is either A or C,
and N can be A, C, G or T (SEQ ID NO 14). The light
chain library formed from this amplification was
designated CDR3-LCNC10. The primer pool designated
p313K316OVb for incorporating 16 randomized amino acid
residues had the noncoding nucleotide sequence written
in the 5' to 3' direction,

5' GTTCCACCTTGGTCCCTTGGCCGAAMNNMNNMNNMNNMNNMNNMNNMNNMNNMNNMNN
MNNMNNMNNMNNMNNMNNMNNMNNMNNACAGTAGTACTGCAAAATC3', where M
is either A or C, and N can be A, C, G or T (SEQ ID NO
15). The light chain library formed from this
amplification was designated CDR3-LCNC16.

Three separate first PCR amplifications were then performed with the KEF primer paired with each of the three noncoding degenerate primers listed above. The amplifications were performed as described in Example 2A1).

The second PCR amplification resulted in the amplification of the light chain from the 5' end of framework region 4 extending to the end of light chain constant region. The 5' coding oligonucleotide, designated p313KF40F, had the nucleotide sequence 5'TTCGGCCAAGGGACCAAGGTGGAAC3' (SEQ ID NO 16). This primer began at the 5' end of framework region 4 providing an overlapping region with the corresponding region in the degenerate oligonucleotide primers. The

3' noncoding primer, T7B, hybridized to the coding strand at the 3' end of the light chain constant domain having the sequence 5'AATACGACTCACTATAGGGCG3' (SEQ ID NO 6). The second PCR reaction was performed as described above.

For overlap PCR, 100 ng of the amplification products from the first and second reactions were pooled following purification and a third round of PCR was performed using the primer pair, KEF and T7B, as described above to form a complete light chain fragment by overlap extension. The light chain fragment amplification products from 15 parallel reactions were first pooled and then gel purified as described above prior to their incorporation into the pC3AP313 surface display phagemid expression vector to form a library as described in Example 4A. The resultant semisynthetic light chain libraries encoded a CDR3 of 8, 10 or 16 randomized amino acids.

The formulations for the various light chain oligonucleotide primers based on the individual oligonucleotide primers presented herein are shown in the Claims and have the corresponding SEQ ID Nos from 26 to 31.

B. Preparation of Randomized Sites Within the Heavy Chain CDR3 of a Phagemid Fab Display Protein Produced by a Dicistronic Expression Vector

Heavy chain libraries having randomized CDR3 in lengths of 5, 10 and 16 amino acids were also prepared using the pC3AP313 surface display expression vector as the PCR template. The resultant libraries prepared as described below were then crossed with the K8, K9 and K10 light chain libraries prepared in Example 2A1). The heavy chain CDR3 (HCDR3) having 10

amino acid residues is approximately the average length utilized in human antibodies. CDR3 having 5 and 16 amino acid residues were chosen to be representative of short and long CDRs respectively based on a previous report on the genetic diversity in this region. Complete randomization using an NNK or NNS degeneracy yielded libraries designated 5, 10 and 16.

Alternatively, the penultimate position of the HCDR3 was fixed as aspartic acid yielding libraries designated G, F and E, respectively, 5, 10 and 16 amino acid residue CDR3s. The first position of the F and E libraries was also fixed as a glycine residue encoded by the triplet codon GGT. The penultimate aspartic acid, Kabat position 101, is conserved in 75% of human antibodies as described by Kabat et al., supra, the disclosure of which is hereby incorporated by reference. The Kabat 101 position is thought to be structurally significant in stabilizing the immunoglobulin loop structure as described by Chothia et al., J. Mol. Biol., 196:901-917 (1987), the disclosure of which is hereby incorporated by reference.

The following amplifications were performed for preparing heavy chain G, F and E libraries. The first PCR reaction resulted in the amplification of the region of the heavy chain fragment in the pC3AP313 phagemid beginning at framework region 1 and extending to the end of framework region 3 which was located 5' to CDR3. The degenerate primer pools designed for use with the pC3AP313 template resulted in the retention of a conserved aspartic acid residue in the next to last position in the CDR3 for all 3 lengths of CDR3s prepared. The retention of the aspartic acid residue in this position is preferred for use in this

invention as the expressed proteins containing this residue exhibit high affinity binding characteristics.

To amplify the 5' end of the heavy chain from framework 1 to the end of framework 3, the following primer pairs were used. The 5' coding oligonucleotide primer, FTX3, having the nucleotide sequence 5'GCAATTAACCCTCACTAAAGGG3' (SEQ ID NO 17), hybridized to the noncoding strand of the heavy chain corresponding to the region 5' of and including the beginning of framework 1. The 3' noncoding oligonucleotide primer, BFR3U, having the nucleotide sequence 5'TCTCGCACAGTAATACACGGCCGT3' (SEQ ID NO 18), hybridized to the coding strand of the heavy chain corresponding to the 3' end of the framework 3 region. The oligonucleotide primers were synthesized by Operon Technologies.

The PCR reaction was performed as described in Example 2A1). The resultant PCR amplification products were then gel purified as described and used in an overlap extension PCR reaction with the products of the second PCR reaction, both as described below, to recombine the two products into reconstructed heavy chains containing mutagenized CDR3s.

The second PCR reaction resulted in the amplification of the heavy chain from the 3' end of framework region 3 extending to the end of C_H1 region. To amplify this region for encoding a 5 random amino acid residue sequence having an aspartic acid in the fourth position in the CDR3, the following primer pairs were used. The 5' coding oligonucleotide primer pool, designated HCDRD5, had the nucleotide sequence represented by the formula, 5'GCCGTGTATTACTGTGCGAGANNKNNKNNKGACNNKTGGGGCCAAGGGACCA CGGTC3' (SEQ ID NO 19), where N can be A, C, G, or T and K is either G or T. The 5' end of the primer pool

is complementary to the 3' end of framework 3 represented by the complementary nucleotide sequence of the oligonucleotide primer BFR3U and the 3' end of the primer pool is complementary to the 5' end of framework 4. The region between the two specified ends of the primer pool is represented by a 12-mer degeneracy of 4 NNK triplets plus a sequence encoding a conserved aspartic acid residue one position from the end of the CDR3. The 3' noncoding oligonucleotide primer, R3B, having the nucleotide sequence 5'TTGATATTCACAAACGAATGG3' (SEQ ID NO 20), hybridized to the coding strand of the heavy chain corresponding to the 3' end of C_H1.

The sequence 5'-NNK-3' represents the coding strand sequence having the complementary sequence 3'-NNM-5' in the primer as read from the 3' to 5' direction. Thus, in the primer as listed below the noncoding strand sequence is 5'-MNN-3' as read in the 5' to 3' direction. The coding triplet sequence 5'-NNK-3' was designed to prevent the production of deleterious stop codons. The only stop codon that could result from the expression of NNK would be an amber mutation that is suppressed when the phagemid is expressed an amber-suppressing host cell, preferably E. coli supE strain.

The second PCR reaction was then performed on the pC3AP313 in an 100 ul reaction as described above containing 1 ug of each of oligonucleotide primers HCDRD5 and R3B. The resultant PCR products encoded a diverse population of mutagenized CDR3s of 5 amino acid residues in length with a conserved aspartic acid residue in the fourth amino acid residue position in the CDR3. The products were then gel purified as described above.

One hundred nanograms of gel purified products

from the first and second PCR reactions were then admixed with 1 ug each of FTX3 and R3B oligonucleotide primers as a primer pair in a final PCR reaction to form a complete heavy chain fragment by overlap extension. The PCR reaction admixture also contained 10 ul 10X PCR buffer, 1 ul Taq polymerase and 8 ul 2.5 Mm DNTP'S as described above. The PCR reaction was performed as previously described.

To obtain sufficient quantities of amplification product, 15 identical PCR reactions were performed. The resulting heavy chain fragments began at framework 1 and extended to the end of C_H1 and had a randomly mutagenized CDR3 for encoding 5 amino acid residues with a conserved aspartic acid residue. The heavy chain fragment amplification products from the 15 reactions were first pooled and then gel purified as described above prior to their incorporation into a digested pC3AP313 surface display phagemid expression vector to form a library as described in Example 4B. The resulting CDR3-randomized heavy chain phagemid library was designated library G.

In addition to randomizing the CDR3 in pC3AP313 for expressing 5 amino acid residues, PCR amplifications were performed for expressing a CDR3 containing 10 amino acid residues. Two separate PCR amplifications were performed as described above with the only exception being that, in the second reaction, the 5' coding degenerate primer, designated HCDRD10, used to encode 10 amino acid residues comprising the heavy chain CDR3. The degenerate 5' coding primer used here was designed to retain the first amino acid position of a glycine residue in the pC3AP313 template and incorporate a conserved aspartic acid residue in the ninth amino acid position. The HCDRD10 primer had the formula:

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library was designated library E.

As described above, the resultant randomized heavy chain CDR3s of various lengths having a conserved aspartic acid residue in the penultimate position amplified from pC3AP313 were purified, digested and ligated back into pC3AP313 for preparation of separate expression libraries as described in Example 4B.

In similar overlap PCR amplifications, heavy chain libraries having completely randomized CDR3s in lengths of 5, 10 or 16 were prepared. The degenerate oligonucleotide pool for preparing the CDR3-HC5 library had the nucleotide formula

5'GTGTATTATTGTGCGAGANNSNNSNNSNNSNNSNSTGGGGCCAAGGGACCACG3', where N can be either A, C, G or T and S is either G or C (SEQ ID NO 23). The resultant library was designated CDR3-HC5. The degenerate oligonucleotide pool for preparing the CDR3-HC10 library had the nucleotide formula

5'GTGTATTATTGTGCGAGANNSNNSNNSNNSNNSNNSNNSNNSNNSNSTGGGGCCAAGGGACCACG3', where N can be either A, C, G or T and S is either G or C (SEQ ID NO 24). The resultant library was designated CDR3-HC10. The degenerate oligonucleotide pool for preparing the CDR3-HC16 library, designated 7ECDR3, had the nucleotide formula 5'GTGTATTATTGTGCGAGANNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNSTGGGGCCAAGGGACCACG3', where N can be either A, C, G or T and S is either G or C (SEQ ID NO 25). The resultant library was designated CDR3-HC16.

As described above, the resultant completely randomized heavy chain CDR3s of various lengths amplified from pC3AP313 were then purified, digested and ligated back into a digested pC3AP313 expression vector for preparation of an expression library as described in Example 4B.

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3. Preparation of Heavy and Light Chain Expression
Vector Libraries Having a Universal Light Chain

A. Crossed Random Heavy Chain Libraries with a
Universal Light Chain

In order to obtain expressed human Fab
antibody libraries comprised of a population of random
heavy chain fragments and a single universal light
chain, crossed phagemid libraries are constructed.

The libraries provide for the expression of
recombinant human Fab antibodies having a population
of random heavy chains and a single universal light
chain for selection of Fab antibodies that bind
preselected ligands with high affinity. Libraries in
which heavy chains are random are prepared as
described in Barbas, et al., Proc. Natl. Acad. Sci.
USA, 88:7978-7982 (1991). The pC3AP313 vector
containing a universal light chain is digested with
Xho I and Spe I to remove the pC3AP313 natural heavy
chain and replace it with Xho I and Spe I digests of
the random heavy chain library. Alternatively,
libraries in which heavy chains are random are
prepared by digestion of the p6F vector described in
Example 8 containing a different universal light chain
with Xho I and Spe I to remove the p6F natural heavy
chain and replace it with Xho I and Spe I digests of a
random heavy chain library. To verify the presence of
random heavy chains and a universal light chain,
randomly selected clones from each crossed library are
sequenced.

B. Crossed Randomized CDR Heavy Chain Libraries
with Universal Light Chain

Alternatively, expressed human Fab antibody
libraries comprised of a population of randomized CDR

heavy chain fragments and a single universal light chain, can also be obtained by the construction of crossed phagemid libraries. The libraries provide for the expression of recombinant human Fab antibodies having randomized CDR heavy chains and a single universal light chain for the selection of Fab antibodies that bind preselected ligands with high affinity.

Libraries in which the CDR3 region of the heavy chain is randomized are prepared as described in Example 4B. Alternatively, the CDR1 or CDR2 region of the heavy chain is randomized by the methods taught in Example 4B. In addition, a library of heavy chains having one or more randomized CDR region created to generate even greater diversity of the heavy chain CDR regions is contemplated. The pC3AP313 vector containing the universal light chain is digested with Xho I and Spe I to remove the pC3AP313 natural heavy chain and the Xho I and Spe I digests of the randomized heavy chain libraries are combined randomly (crossed) into the digested pC3AP31V vector to form a population of vectors having the universal light chain and one of the randomized heavy chains from the heavy chain library. Crossed libraries are thus prepared by the combination of a universal light chain with a randomized heavy chain library. To verify the presence of randomized heavy chains and a single universal light chain, randomly selected clones from each crossed library are sequenced.

4. Preparation of Heavy and Light Chain Expression Vector Libraries Having Randomized CDR3

A. Light Chain Libraries

The light chains having randomized CDR3 from

the overlap PCR amplifications using both coding and noncoding degenerate oligonucleotide primers produced in Example 2A were then separately introduced into the pC3AP313 Pcomb3-based monovalent Fab phage display vector prepared as described in Example 1. The PCR products resulting from each of the amplifications prepared in Example 2A were separately inserted into a phagemid expression vector to prepare phagemid libraries. As described below, the resultant gel purified light chain PCR CDR3-randomized products prepared in Example 2A were digested with restriction enzymes and separately ligated into the pC3AP313 phagemid expression vector that was similarly digested.

For preparation of phagemid libraries for expressing the light chain PCR products prepared in Example 2A, the PCR products were separately digested with Sac I and Aat II and separately ligated with a similarly digested pC3AP313 phagemid expression vector prepared as described in Example 1. Digestion of the pC3AP313 vector with Sac I and Aat II removed the nucleotide sequence region beginning at the 5' end of the native light chain variable domain to the beginning of framework 4. The ligation thus resulted in operatively linking the light chain framework 1 through randomized CDR3 PCR products with the native framework 4 domain present in the pC3AP313 vector. The expression of the resultant light chain libraries was under the control of a LacZ promoter and pelB leader sequence.

Phagemid libraries for expressing each of the Fabs having randomized light chain CDR3 of this invention were prepared in the following procedure. To form circularized vectors containing the PCR product insert, 640 ng of the digested PCR products

was admixed with 2 ug of the linearized pC3AP313 phagemid vector and ligation was allowed to proceed overnight at room temperature using 10 units of BRL ligase (Gaithersburg, MD) in BRL ligase buffer in a reaction volume of 150 ul. Five separate ligation reactions were performed to increase the size of the phage library having randomized CDR3. Following the ligation reactions, the circularized DNA was precipitated at -20C for 2 hours by the admixture of 2 ul of 20 mg/ml glycogen, 15 ul of 3 M sodium acetate at Ph 5.2 and 300 ul of ethanol. DNA was then pelleted by microcentrifugation at 4C for 15 minutes. The DNA pellet was washed with cold 70% ethanol and dried under vacuum. The pellet was resuspended in 10 ul of water and transformed by electroporation into 300 ul of *E. coli* XL1-Blue cells to form a phage library. The total yield from the PCR amplification and transformation procedure described herein was approximately 10^8 independent transformants.

The light chain libraries having randomized CDR3 of 4, 5, 6 and 10 amino acid residues (respectively in a CDR3 of 8, 9, 10 and 10 amino acid residues) resulting from the PCR products obtained with the coding degenerate primer pool were respectively designated K8, K9, K10 and K10'. The light chain libraries having CDR3 of 8, 10 and 16 amino acid residues resulting from the PCR products obtained with the noncoding degenerate primer pool were respectively designated CDR3-LCNC8, CDR3-LCNC10 and CDR3-LCNC16.

B. Heavy Chain Libraries

The heavy chains having randomized CDR3 produced in Example 2B from overlap PCR amplifications were then separately introduced into the monovalent Fab phage display vector Pcomb3 prepared as described

in Example 1. The PCR products resulting from each of the amplifications prepared in Example 2B were separately inserted into a phagemid expression vector to prepare phagemid libraries. As described below, the resultant gel purified light chain PCR fragments prepared in Example 2B were digested with the restriction enzymes and separately ligated into the pC3AP313 phagemid expression vector that was similarly digested.

For preparation of phagemid libraries for expressing the heavy chain PCR products prepared in Example 2B, the PCR products were digested with Xho I and Spe I and separately ligated with a similarly digested pC3AP313 phagemid expression vector prepared as described in Example 1. Digestion of the pC3AP313 vector with Xho I and Spe I removed the native nucleotide sequence region beginning at the 5' end of the heavy chain variable domain to the beginning of the heavy chain constant domain, C_H1. The ligation thus resulted in operatively linking the framework 1 through randomized CDR3 PCR products with the native C_H1 domain present in the pC3AP313 vector. The expression of the resultant heavy chain libraries was under the control of a LacZ promoter and pelB leader sequence.

Phagemid libraries for expressing each of the Fabs having randomized heavy chain CDR3 of this invention were prepared as described above for the light chain. The total yield from the PCR amplification and transformation procedure described herein was approximately 10⁸ independent transformants.

The heavy chain libraries with CDR3 of 5, 10 or 16 amino acid residues in length resulting from the PCR products obtained retaining an aspartic acid in

the penultimate position were respectively designated G, F and E. The heavy chain libraries with completely randomized CDR3 of 5, 10 or 16 amino acid residues in length were respectively designated CDR3-HC5, CDR3-HC10 and CDR3-HC16.

C. Crossed Heavy and Light Chain Libraries

In order to obtain expressed human Fab antibodies having both randomized heavy and light chain fragments, crossed phagemid libraries were constructed. The libraries provided for the expression of recombinant human Fab antibodies having heavy and light chains in which the CDR3 in both were selectively randomized for selection of Fab antibodies that bind synthetic haptens with high affinity. Libraries in which both CDR3s were randomized were prepared by digestion of the light chain libraries prepared in Example 4A with Xho I and Spe I to remove the pC3AP313 natural heavy chain and replace it with Xho I and Spe I digests of the synthetic heavy chain libraries prepared in Example 4B. Nine crossed libraries were prepared by combination of K8, K9 and K10 light chain libraries with the G, F and E heavy chain libraries. In addition, to examine the role of the light chain CDR3, the heavy chain domain of a previously selected clone that encoded a Fab antibody, designated F22, that reacted with fluorescein was crossed with the light chain K8, K9 and K10 libraries. Crossed libraries were designated by listing the light chain library first separated from the heavy chain library by a slash, e.g., K8/F. All resultant crossed libraries consisted of at least 10^8 independent transformants except for K9/F22 and K8/F22 that contain 10^7 transformants. The crossed library designated K10/E consisted of Fab fragments were 20

positions were randomized. In order for the crossed libraries to be "complete", i.e., where all possible members (combinations of heavy and light chain library members) are represented, more than 10^{30} transformants would be necessary. To verify the targeted mutagenesis of the light and heavy chain CDR3, randomly selected clones from each uncrossed library were sequenced prior to crossing.

The other light chain libraries, K10', CDR3-LCNC8, CDR3-LCNC10 and CDR3-LCND16 are similarly crossed with all of the heavy chain libraries prepared in Example 4B to form additional crossed libraries having varying lengths of CDR3 having varying randomized amino acid residues.

D. Crossed CDR3 Randomized Heavy Chain and A Single Universal Light Chain Libraries

In order to obtain expressed human Fab antibodies having randomized heavy and universal light chain fragments, crossed phagemid libraries are constructed. The libraries provide for the expression of recombinant human Fab antibodies having heavy chains in which the CDR3 are randomized for the selection of Fab antibodies that bind preselected ligands with high affinity. The libraries also provide for the expression of recombinant human Fab antibodies having a single universal light chain for the selection of Fab antibodies that bind preselected ligands with high affinity. Libraries in which CDR3 of the heavy chain are randomized, are prepared by digestion of the universal light chain with Xho I and Spe I to remove the pC3AP313 natural heavy chain and replace it with Xho I and Spe I digests of the synthetic heavy chain libraries prepared in Example 4B. Crossed libraries are prepared by combination of

a universal light chain, with the amino acid sequence as shown in SEQ NO 2, with the G, F and E heavy chain libraries. The crossed library consists of Fab fragments where 5, 10, or 16 positions of the heavy chain CDR3 are randomized and a single universal light chain. To verify the targeted mutagenesis of the heavy chain CDR3, randomly selected clones from each uncrossed library are sequenced prior to crossing.

Another universal light chain, 6F with the nucleotide sequence as shown in SEQ ID NO 62, is similarly crossed with all of the heavy chain libraries prepared in Example 4B to form additional crossed libraries having varying lengths of heavy chain CDR3 with varying randomized amino acid residues and a single universal light chain.

5. Selection of Anti-Hapten Fab Antibodies Expressed on Phage

A. Preparation of Phage Expressing Semisynthetic Fab Heterodimers

After transformation, to isolate phage expressing Fabs reactive with synthetic haptens, panning on target synthetic haptens was performed as described in Example 5B below.

Phage were first prepared on which the semisynthetic Fab antibodies were expressed for selecting on synthetic haptens. Three ml of SOC medium (SOC was prepared by admixture of 20 grams (g) bacto-tryptone, 5 g yeast extract and 0.5 g NaCl in 1 liter of water, adjusting the Ph to 7.5 and admixing 20 ml of glucose just before use to induce the expression of the heavy chain domain anchored to the phage coat protein 3 (Fd-cpIII) and soluble light chain heterodimer) were admixed to selected phage

libraries and the culture was shaken at 220 rpm for 1 hour at 37C. Then 10 ml of SB (SB was prepared by admixing 30 g tryptone, 20 g yeast extract, and 10 g Mops buffer per liter with Ph adjusted to 7) containing 20 ug/ml carbenicillin and 10 ug/ml tetracycline were admixed and the admixture was shaken at 300 rpm for an additional hour. This resultant admixture was admixed to 100 ml SB containing 50 ug/ml carbenicillin and 10 ug/ml tetracycline and shaken for 1 hour, after which helper phage VCSM13 (10^{12} pfu) were admixed and the admixture was shaken for an additional 2 hours. After this time, 70 ug/ml kanamycin was admixed and maintained at 30C overnight. The lower temperature resulted in better heterodimer incorporation on the surface of the phage. The supernatant was cleared by centrifugation (4000 rpm for 15 minutes in a JA10 rotor at 4C). Phage were precipitated by admixture of 4% (w/v) polyethylene glycol 8000 and 3% (w/v) NaCl and maintained on ice for 30 minutes, followed by centrifugation (9000 rpm for 20 minutes in a JA10 rotor at 4C). Phage pellets were resuspended in 2 ml of PBS and microcentrifuged for three minutes to pellet debris, transferred to fresh tubes and stored at -20C for subsequent screening as described below.

For determining the titering colony forming units (cfu), phage (packaged phagemid) were diluted in SB and 1 ul was used to infect 50 ul of fresh ($A_{OD600} = 1$) E. coli XL1-Blue cells grown in SB containing 10 ug/ml tetracycline. Phage and cells were maintained at room temperature for 15 minutes and then directly plated on LB/carbenicillin plates.

B. Selection of the Phagemid-Displayed Semisynthetic Fab Heterodimers

alternating 3% BSA and 2% non-fat dry milk blocking of the microtiter dish at each round of selection.

Wells of a microtiter plate (Costar 3690) were separately coated overnight at 4C with the purified target conjugates prepared above. The wells were washed twice with water and blocked by completely filling the well with 3% (w/v) bovine serum albumin (BSA) in PBS and incubating the plate at 37C for 1 hour. Blocking solution was removed by shaking, 50 ul of each of the phage libraries prepared above (typically 10^{11} cfu) were added to each well, and the plate was incubated for 2 hours at 37C.

Phage were removed and the plate was washed once with water. Each well was then washed 10 times with TBS/Tween (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% Tween 20) over a period of 1 hour at room temperature then pipetted up and down to wash the well, each time allowing the well to remain completely filled with TBS/Tween between washings. The plate was washed once more with distilled water and adherent phage were eluted by the addition of 50 ul of elution buffer (0.1 M Hcl, adjusted to Ph 2.2 with solid glycine, containing 1 mg/ml BSA) to each well and incubation at room temperature for 10 minutes. The elution buffer was pipetted up and down several times, removed, and neutralized with 3 ul of 2 M Tris base per 50 ul of elution buffer used.

Eluted phage were used to infect 2 ml of fresh ($OD_{600} = 1$) E. coli XL1-Blue cells for 15 minutes at room temperature, after which 10 ml of SB containing 20 ug/ml carbenicillin and 10 ug/ml tetracycline was admixed. Aliquots of (20, 10, and 1/10 ul were removed for plating to determine the number of phage (packaged phagemids) that were eluted from the plate. The culture was shaken for 1 hour at 37C, after which

1) Multiple Pannings of the Phage Library
Having Phagemid Fab-Displayed Synthetic
Binding Site Proteins

The phage libraries produced in Example
5 4A, 4B and 4C were panned as described herein on
microtiter plates coated with the synthetic hapten
conjugate target molecules. Three synthetic haptens
were chosen for screening for improved high affinity
antibodies having either a randomized heavy or light
10 chain domain or both. The conjugates, shown in Figure
1 and labeled as 1, 2, and 3, respectively, were
fluorescein-BSA (Fl-BSA), S-BSA, an analog for the
selection of catalytic antibodies that catalyze a
decarboxylation reaction, and C-BSA, similar to the
15 other two haptens but containing a flat aromatic ring
system and lacking the anionic character of the other
haptens. Conjugate 1 was described by Barbas et al.,
Proc. Natl. Acad. Sci., USA, 89:4457-4461 (1992), the
disclosure of which is hereby incorporated by
20 reference. Conjugates 2 and 3 have been previously
described by Lewis et al., Reports, 1019-1021 (1991),
the disclosure of which is hereby incorporated by
reference. The reagents were used at a concentration
of 40 ug/ml in the coating buffer, 0.1 M bicarbonate
25 at Ph 8.6.

The panning procedure described was a
modification of that originally described by Parmley
et al., Gene, 73:305-318 (1988). This procedure,
described below for one preparation, was followed for
30 each of the phage preparations for all libraries
prepared for use in this invention. Since the haptens
were conjugated to BSA, selective pressure was applied
to select for hapten binding and against BSA binding.
This was accomplished by resuspending phage in TBS
35 containing 1% BSA prior to selection and by

alternating 3% BSA and 2% non-fat dry milk blocking of the microtiter dish at each round of selection.

Wells of a microtiter plate (Costar 3690) were separately coated overnight at 4C with the purified target conjugates prepared above. The wells were washed twice with water and blocked by completely filling the well with 3% (w/v) bovine serum albumin (BSA) in PBS and incubating the plate at 37C for 1 hour. Blocking solution was removed by shaking, 50 ul of each of the phage libraries prepared above (typically 10^{11} cfu) were added to each well, and the plate was incubated for 2 hours at 37C.

Phage were removed and the plate was washed once with water. Each well was then washed 10 times with TBS/Tween (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% Tween 20) over a period of 1 hour at room temperature then pipetted up and down to wash the well, each time allowing the well to remain completely filled with TBS/Tween between washings. The plate was washed once more with distilled water and adherent phage were eluted by the addition of 50 ul of elution buffer (0.1 M Hcl, adjusted to Ph 2.2 with solid glycine, containing 1 mg/ml BSA) to each well and incubation at room temperature for 10 minutes. The elution buffer was pipetted up and down several times, removed, and neutralized with 3 ul of 2 M Tris base per 50 ul of elution buffer used.

Eluted phage were used to infect 2 ml of fresh ($OD_{600} = 1$) E. coli XL1-Blue cells for 15 minutes at room temperature, after which 10 ml of SB containing 20 ug/ml carbenicillin and 10 ug/ml tetracycline was admixed. Aliquots of (20, 10, and 1/10 ul were removed for plating to determine the number of phage (packaged phagemids) that were eluted from the plate. The culture was shaken for 1 hour at 37C, after which

it was added to 100 ml of SB containing 50 ug/ml carbenicillin and 10 ug/ml tetracycline and shaken for 1 hour. Helper phage VCSM13 (10^{12} pfu) were then added and the culture was shaken for an additional 2 hours. After this time, 70 ug/ml kanamycin was added and the culture was incubated at 37C overnight. Phage preparation and further panning were repeated as described above.

Following each round of panning, the percentage yield of phage were determined, where % yield - (number of phage eluted/number of phage applied) X 100.

The final phage output ratio was determined by infecting 2 ml of logarithmic phase XL1-Blue cells as described above and plating aliquots on selective plates. Following the washing and acid elution from the first round of panning, the phage-displayed Fab libraries were then combined in subsequent rounds of panning to identify by competitive binding the highest affinity clones from the collection of libraries. By sequencing the selected binders, the source library of the clones was then determined.

From this procedure, clones were selected from each of the Fab libraries for their ability to bind to their respective selected synthetic targets. The panned phage surface libraries were then converted into ones expressing soluble semisynthetic Fab antibodies for further characterization as described in Example 5C.

C. Preparation of Soluble Fab-Displayed Binding Site Proteins

In order to further characterize the specificity of the semisynthetic Fab antibodies expressed on the surface of phage as described above,

soluble heterodimers were prepared and analyzed in ELISA assays on synthetic conjugate target-coated plates and by competitive ELISA with increasing concentrations of soluble competitor protein as described below.

To prepare soluble Fabs consisting of heavy and light chains (i.e., heterodimers), phagemid DNA from positive clones selected in Example 5B above was isolated and digested with Spe I and Nhe I. Digestion with these enzymes produced compatible cohesive ends. The 4.7 kb DNA fragment lacking the gIII portion was gel-purified (0.6% agarose) and self-ligated. Transformation of E. coli XL1-Blue afforded the isolation of recombinants lacking the gIII fragment. Clones were examined for removal of the gIII fragment by Xho I/Xba I digestion, which should yield an 1.6 kb fragment. Clones were grown in 100 ml SB containing 50 ug/ml carbenicillin and 20 Mm MgCl₂ at 37C until an OD₆₀₀ of 0.2 was achieved. IPTG (1 Mm) was added and the culture grown overnight at 30C (growth at 37C provides only a light reduction in heterodimer yield). Cells were pelleted by centrifugation at 4000 rpm for 15 minutes in a JA10 rotor at 4C. Cells were resuspended in 4 ml PBS containing 34 ug/ml phenylmethylsulfonyl fluoride (PMSF) and lysed by sonication on ice (2-4 minutes at 50% duty). Debris was pelleted by centrifugation at 14,000 rpm in a JA20 rotor at 4C for 15 minutes. The supernatant was used directly for ELISA analysis and was stored at -20C. For the study of a large number of clones, 10-ml cultures provided a sufficient amount of the semisynthetic Fab antibodies for analysis. In this case, sonications were performed in 2 ml of buffer.

The soluble heterodimers prepared above were

assayed by ELISA where applicable as described in Example 6.

6. Characterization of Soluble Semisynthetic Fab
Heterodimers

A. ELISA

Preliminary ELISA assays were performed to first characterize the binding specificity of the panned phage semisynthetic Fab antibodies prepared above toward synthetic haptens. For ELISA, 1 ug/well of the synthetic haptens prepared in Example 5B was separately admixed to individual wells of a microtiter plate and maintained at 4C overnight to allow the hapten solution to adhere to the walls of the well. After the maintenance period, the wells were washed once with PBS and thereafter maintained with a solution of 3% BSA to block nonspecific sites on the wells. The plates were maintained at 37C for 1 hour after which time the plates were inverted and shaken to remove the BSA solution. Soluble Fab heterodimers expressing the semisynthetic Fab heterodimers prepared in Example 5C were then admixed separately to each well and maintained at 37C for 1 hour to form a immunoreaction products. Following the maintenance period, the wells were washed 10 times with PBS to remove unbound soluble antibody and then maintained with a secondary goat anti-human FAB conjugated to alkaline phosphatase diluted in PBS containing 1% BSA. The wells were maintained at 37C for 1 hour after which the wells were washed 10 times with PBS followed by development with p-nitrophenyl phosphate.

Following 5 rounds of selection as described in Example 5B and conversion of the phagemid from surface display form to soluble antibody producing form, 20 of

20 clones selected for binding the fluorescein conjugate (1), 18 of 20 selected for binding conjugate S-BSA (2) and 1 of 20 selected for binding conjugate C-BSA (3) were positive in ELISA analysis. All clones from F22-derived libraries were also positive following selection for binding to conjugate 1.

Cross reactivities of purified clones were examined by ELISA and are shown in Figure 2. The antigens used in the ELISA shown from left to right in Figure 2 are the original pC3AP313-specific tetanus toxoid (forward slashed bar), Fl-BSA conjugate (black bar), BSA (horizontal bar), S-BSA conjugate (backward slashed bar) and C-BSA conjugate (white bar). Clones F22, P2, S4, and S10 were specific for the conjugate on which they were selected. Clone S4 retained some reactivity to the parent antigen tetanus toxoid. Clones S2 and C15 were more promiscuous in binding. Selection against binding to BSA was effective as indicated by the limited reactivity of the Fab to this antigen.

B. Affinity Characterization

The affinities of several purified clones were examined by surface plasmon resonance. Only observed monomeric Fab as judged by gel filtration has been observed in contrast to a recent report of single-chain antibody dimerization as described by Griffiths et al., EMBO J., 12:725-734 (1993). The determination of on and off affinity constants, respectively, k_{on} and k_{off} , for selected clones were performed using the Biacore instrument from Pharmacia Biosensor (Piscataway, NJ, according to manufacturer's instructions. The Fl-BSA conjugate was immobilized in 10 Mm acetate buffer at Ph 2.5 to yield 600 resonance units on a CM5 Biacore sensor chip. The k_{on} and k_{off}

were determined by standard analysis in PBS at flow rates of 5 and 8 ul/minutes, respectively as described by Altschun et al., Biochem., 31:6298-6304 (1992).

A compilation of kinetic and equilibrium constants is given in Table I. All K_d 's approached the nanomolar range. Clone P2 which was strongly selected from F22 derived libraries had a slightly lower affinity than the parent clone. The affinity of F22 for Fl-BSA conjugate by surface plasmon resonance is in close agreement with affinity as determined by competitive analysis.

Table 1

Clone	$k_{on}(M^{-1}s^{-1})$	$k_{off}(s^{-1})$	$K_a(M^{-1})$	$K_d(nM)$
F22	6.4×10^5	2.2×10^{-2}	2.9×10^7	34
P2	2.0×10^5	1.6×10^{-2}	1.3×10^7	80
S2	2.8×10^5	8.0×10^{-3}	3.5×10^7	29
S4	4.0×10^5	2.2×10^{-2}	1.8×10^7	56
S10	3.5×10^5	1.3×10^{-2}	2.7×10^7	37

C. Sequence Determination of the Binding Site Proteins

Nucleic acid sequencing was performed on double-stranded DNA using Sequenase 1.0 (USB, Cleveland, OH) encoding the specific soluble synthetic hapten-binding Fab heterodimers of this invention characterized above.

The sequences of the CDR3 regions from the selected antibodies are shown in Table 2 and 3. On the left hand side of both tables, the selected antibodies (referred to as the clone) and the anti-hapten conjugate number, 1, 2 or 3, on which the antibody was screened, are listed. The next column from left to right shown is either the amino acid

residue sequence of the heavy (HCDR3 in Table 2) and light chain CDR3 (LCDR3 in Table 3) from the designated clone. The SEQ ID Nos are listed adjacent to each of the heavy and light chain sequences. The last column in each table shows the designation of the crossed light and heavy chain library from which the clone was derived and selected. In all cases, the light chain is listed first followed by the heavy chain library or none if applicable.

Table 2

<u>Clone/Conjugate</u>	<u>HCDR3</u>	<u>SEQ ID NO</u>	<u>Library</u>
FL3/1	GWSRWSGLDW	32	K10/F
FL18/1	SSTKIMRLDT	33	K9/F
FL19/1	GMFRRGFYDR	34	F
FL12/1	GVRNNFGRWHVWDS	35	E
FL13/1	GRAVRGSRKRVLYDR	36	E
FL15+1/1	GRPGVVRRIAPRMDI	37	K9/E
FL17/1	GPKGVFPRWGMASFDR	38	K10/E
F22/1	GVNLFRVRNSRPHLDM	39	16
P2/1	GVNLFRVRNSRPHLDM	39	K9/F22
P3/1	GVNLFRVRNSRPHLDM	39	K9/F22
P4/1	GVNLFRVRNSRPHLDM	39	K9/F22
P5/1	GVNLFRVRNSRPHLDM	39	K10/F22
P6/1	GVNLFRVRNSRPHLDM	39	K10/F22
P7/1	GVNLFRVRNSRPHLDM	39	K10/F22
	GVNLFRVRNSRPHLDM	39	K10/F22
S4/2	GLRGSRGFDR	40	K10/F
S10/2	GSWLRGPYDM	41	
S12/2	GTLGEGGYDR	42	K10/F
S2/2	GWRSSRGVVWFSGDA	43	K10/E
C13/3	GDWGWFTRVATWRPDV	44	K10/E

Table 3

<u>Clone/Conjugate</u>	<u>LCDR3</u>	<u>SEQ ID NO</u>	<u>Library</u>
FL3/1	QQYLPGGRYT	45	K10/F
FL18/1	QQYRVEGQT	46	K9/F
FL19/1	QQYGGSPW	47	F
FL12/1	QQYGGSPW	47	E
FL13/1	QQYGGSPW	47	E
FL15+1/1	QQYSRHRFT	48	K9/E
FL17/1	QQYRYPLIWT	49	K10/E
F22/1	QQYGSSLWT	50	16
P2/1	QQYTRPGVT	51	K9/F22
P3/1	QQYSFKNWT	52	K9/F22
P4/1	QQYGYRKWT	53	K9/F22
P5/1	QQYTPRRGAT	54	K10/F22
P6/1	QQYTPRVGHT	55	K10/F22
P7/1	QQYKYGRGMT	56	K10/F22
	QQYKYGRGMT	56	K10/F22
S4/2	QQYGKKQWT	57	K10/F
S10/2	QQYVRRSGT	58	
S12/2	QQYGKRSPVT	59	K10/F
S2/2	QQYARATGLT	60	K10/E
C13/3	QQYSRFVSRT	61	K10/E

A number of features are immediately obvious from looking at the amino acid residue sequence of the selected clones, the libraries from which they were derived and the synthetic hapten on which they were selected. No clones derived from libraries containing HCDR3 length of 5 survived the competitive selection. Furthermore, no clones derived from libraries with only light chain variation were selected. All clones were derived from heavy chain libraries where the first and penultimate residues have been fixed as Gly and Asp, respectively. Clone FL18 contained a serine

(S) at the first position that is likely an artifact of the synthesis and assembly and is the result of a single base change (GGT to AGT). This has been noted in previous examinations of libraries E and F. These results indicate that completeness of a semisynthetic Fab library does not necessarily correlate with the quality of antibodies which can be derived from it. Libraries K8, CDR3-HC5, and G all contained sufficient members to be judged as 99% complete and yet no clones from these libraries survived the competitive selection. Indeed most clones were derived from the crossed libraries that were the most incomplete but probably most structurally diverse. These results highlight the fact that an evolved combining site is under remodeling which may be best achieved with more extensive mutation rather than less. This argument may explain the low affinity clones isolated by the randomization of 5 residues reported previously by Hoogenboom et al., J. Mol. Biol., 227:381-388 (1992).

There is evidence for selection of consensus sequence in the clones. For example, in the eighth position of HCDR3 of clones S4, S10, and S12 is an aromatic residue. Their corresponding light chains contain the basic doublets KK, RR, and KR, respectively. Furthermore, sequence similarity is noted in clones S4 and S2 which differ in length but contain very similar carboxy-terminal HCDR3 regions. Clone S10 and S2 were found 3 and 2 times, respectively, identical at the nucleotide level following sequencing of 7 clones.

Examination of the role of LCDR3 in the previously selected clone F22 revealed that considerably different sequence may be tolerated in this region as compared to the starting clone. The predominant clone was P2 that was found 5 times

identical at amino acid level among the 10 clones
sequenced. This clone was found to be encoded by 4
unique nucleotide sequences. Naturally occurring
murine and human kappa light chain CDR3 regions show a
strong conservation of Pro at Kabat position 95. None
of the clones derived from the semisynthetic libraries
contain proline (P) at this position. This indicates
that proline is conserved for something other than
structural reasons or there is editing of this
sequence at some level.

Thus, a variety of anti-hapten semisynthetic Fab
antibodies can be directly selected from semisynthetic
antibody libraries derived from the randomization of 1
or 2 CDR regions, specifically in the heavy and light
chain CDR3. Like naturally occurring antibodies,
semisynthetic antibodies exhibited differing degrees
of cross-reactivity. Libraries with greater
structural diversity, those with more residues
randomized, were functionally superior over complete
but structurally limited libraries. However,
constraining diversity in the heavy chain CDR3 to the
extent of holding the penultimate position fixed as
aspartic acid improved the quality of the library and
highlights the structural role of this residue. No
such phenomena has yet to be observed in the light
chain CDR3 though 4 positions in this region have yet
to be examined.

7. Preparation of a Dicistronic Expression Vector
Library Capable of Expressing a Phagemid Fab
Display Protein Derived From Human Anti-Thyroid
Peroxidase Antibody Light and Heavy Chain
Libraries:

A. Preparation of Lymphocyte MRNA

Thyroid tissue was obtained from a patient with Hashimoto's thyroiditis containing anti-thyroid peroxidase antibodies, and thyroid lymphocytes were isolated from the thyroid tissue, as described in Atherton et al., Immunology, 55:271-279 (1985). RNA was then extracted from the freshly isolated cells (Hexham et al., Autoimmunity, 12:135-141 (1992) and Hexham et al., Autoimmunity, 14:169-172 (1992)). Analysis of the Hashimoto's patient serum by ELISA (Schardt et al., J. Immunol. Methods, 55:155-168 (1982)) at the time of the operation indicated the presence of high levels of thyroid peroxidase (TPO) autoantibodies, primarily of the IgG/kappa type.

B. Construction of Heavy and Light Chain Thyroid Peroxidase Antibody Libraries in Lambda Phage

Heavy and light chain thyroid peroxidase antibody libraries were first constructed in lambda phage as described in Hexham et al., Autoimmunity, 12:135-141 (1992), using the lymphocyte mRNA isolated in Example 7A. The heavy and light chain lambda phage libraries were converted to phagemid libraries through an in vivo excision process (Short et al., supra) using interference resistant M13 helper phage VCSM13 (Stratagene, La Jolla, California).

Following the excision of the lambda phage library encoding the light chain, eleven clones were randomly chosen for further analysis. DNA was isolated and the nucleotide sequence determined by the dideoxy chain-termination method (Sanger et al., Proc. Natl. Acad. Sci. U.S.A., 74:5463-5467 (1977)) using Sequenase 2.0 (United States Biochem).

C. Construction of Heavy and Light Chain
Thyroid Peroxidase Antibody Libraries in
Pcomb3

5 The heavy and light chain antibody encoding
sequences identified in Example 7B were removed from
the excised phagemid vector and inserted into the
monovalent Fab phage display vector, Pcomb3. The
heavy and light chain sequences were respectively
isolated by restriction digestion with Xho I/Spe I and
10 Sac I/Xba I and ligated into a similarly digested
Pcomb3 vector. The ligation procedure in creating
expression vector libraries was performed as described
in Example 2. The primary library contained 10^5
independent clones. Twelve clones were selected at
15 random and analyzed by restriction digestion of the
DNA with Not I. 83% of the clones examined contained
the 2.5 kb insert fragment consistent with an Fab-
containing vector.

20 8. Selection of Anti-Thyroid Peroxidase Fab
Antibodies Expressed on Phage

A. Preparation of Phage Expressing Fab
Heterodimers

25 Phage expressing Fabs reactive with thyroid
peroxidase (TPO) were prepared as described in Example
2 using the expression vector library produced in
Example 7C to form a phage library containing phage
with Fab display protein.

30 B. Selection of the Phagemid-Displayed Fab
Heterodimers

1) Multiple Pannings of the Phage Library
Having Phagemid Fab-Displayed Binding
Site Proteins

The phage library prepared in Example
5 8A was panned as described in Example 5B1 on
microtiter plates coated with TPO target molecules to
isolate phagemid displaying anti-TPO Fab heterodimers.
Consecutive rounds of panning on TPO-coated ELISA
plates resulted in an enrichment of approximately 10^4 -
10 fold. Round 1 of panning gave a recovery of 2×10^3
colony forming units (cfu); round 2 gave a recovery
of 3.2×10^3 cfu; round 3 gave a recovery of $>10^6$
cfu; and round 4 gave a recovery of $>10^7$ cfu. The
panned phage surface expression clones were then
15 converted into clones expressing soluble Fab
antibodies as described in Example 5C for further
characterization.

9. Characterization of Soluble Fab Heterodimers

A. ELISA

ELISA assays were performed to characterize
the binding specificity of individual panned phage Fab
antibodies with TPO. ELISA was conducted as described
25 in Example 6A with TPO instead of the synthetic
haptens as the target molecule and the Fab was
detected with anti-human IgG (Fab) conjugated to
alkaline phosphatase (Sigma, St. Louis, Missouri).

Following 4 rounds of selection as described in
30 Example 8B1 and conversion of the phagemid form from
surface display form to soluble antibody producing
form, 17 of 24 clones selected for binding to TPO were
positive in the ELISA analysis. Cross reactivities of
purified clones with irrelevant proteins were examined
35 by ELISA as described in Example 6A. The antigens

used in the ELISA were a range of concentrations of human TPO, human thyroglobulin (RSR Ltd, Cardiff, CF2 7HE), human myeloperoxidase (Sigma, St. Louis, Missouri), and bovine lactoperoxidase (Sigma, St. Louis, Missouri). Binding of the Fabs to TPO-coated plates was inhibited by human TPO, however, no inhibition was observed with human thyroglobulin (up to 100 Nm), human myeloperoxidase (up to 200 Nm), or bovine lactoperoxidase (up to 10 μ M).

B. Affinity Characterization

The affinities of several purified clones were estimated by inhibition ELISA with various concentrations of TPO as the competitor. The affinity constants of 6F, 7F, and 10I, were estimated to be 8.0×10^8 , 8.0×10^8 , and $0.3 \times 10^9 \text{ M}^{-1}$, respectively.

Thus, three diverse, novel, high-affinity (approximately 10^{-9} M^{-1}) anti-TPO Fab antibodies were directly selected from a Pcomb3 phage display combinatorial library. These Fabs, designated 6F, 7F, and 10I, were obtained with a relative frequency of 12:4:1 from an enriched population of phage with Fab 10I having the highest affinity for TPO.

C. Sequence Determination of the Binding Site Proteins

The nucleotide sequence of the specific soluble TPO-binding Fab heterodimers of this invention was determined. The nucleotide sequence of the anti-TPO monoclonal antibody 2G4 (Horimoto et al., Autoimmunity, 14:1-7 (1992) and Hexham, et al., Autoimmunity, 14:169-172 (1992)) and the SP series of recombinant anti-TPO antibodies (Portolano et al., Biochem. Biophys. Res. Comm., 179:372-377 (1991), Portolano et al., J. Clin. Invest., 90:720-726 (1992),

and Portolano et al., J. Immunol., 150:880-887 (1993))
was also determined. Nucleic acid sequencing was
performed on double-stranded DNA using Sequenase 2.0
(USB, Cleveland, OH). The primers SEQGb, SEQKb, and
the M13 reverse primer were used as described in
Hexham et al., Autoimmunity, 12:135-141 (1992).

Sequence analysis and database searches were
carried out using the SERC Segnet facility on a
Silicon Graphics Crimson running the GCG suite of
programs (Devereux, et al., Nucl. Acids Res., 12:387-
395 (1984)). Variable region sequences were
identified and analyzed using the FASTA program to
search the Genbank and EMBL databases and by direct
comparison with known sequences (Kabat et al., supra).

The sequences of the CDR regions from anti-TPO
antibodies are shown in Tables 4 and 5. On the left
hand side of both tables, the anti-TPO antibodies
(referred to as the clone) are listed. The next
column from left to right shown is either the amino
acid residue sequence of the heavy CDRs (HCDR in Table
4) and light chain CDRs (LCDR in Table 5) from the
designated clone. The SEQ ID NOs corresponding to the
complete amino acid residue sequence as listed in the
Sequence Listing are listed adjacent to each of the
heavy and light chain amino acid sequences in Tables 4
and 5.

Table 4

<u>Clone</u>	<u>HCDR1</u>	<u>HCDR2</u>	<u>HCDR3</u>	<u>SEQ ID NO</u>
10I	SYAMT	SPSANGDFAYYADSVKG	AGRILGVVLWYSLYYGFDV	63
6F	SHDIN	WITNRGTTSTRYAQKFQG	GAGAGGTW	64
SP1.2	GHYMH	WISPNRGATRFAQKFQG	TRTAYYGMDV	65

Table 5

<u>Clone</u>	<u>LCDR1</u>	<u>LCDR2</u>	<u>LCDR3</u>	<u>SEQ ID NO</u>
10I	RASSNISSYIN	AASSLQS	QQSYSTPFT	66
6F	RASQRISYIN	AASSLQS	QQSYSTPYT	67
SP1.2	RASENISSYIN	AASTLQS	QQTYSPPFT	68
SP1.4	RASQTIGTYIN	TASTLQS	QQSYSTPWT	69
SP1.5	RASQNIGKYIN	GTSTLQS	QQSYSTPWT	70

Analysis of the nucleotide and deduced amino acid sequences of the HC and LC variable regions of 6F, 7F, and 10I allows most of the Vkappa VH, JH, Kkappa, and DH genes to be assigned to the germline gene from which they were derived. A striking feature of these antibodies is that five of them (6F, 10I, and the three SP antibodies shown) appear to have VkappaI light chains encoded by the same vk02 or vk012 germline gene (Pargent et al., Eur. J. Immunol., 21:1821-1827 (1991)). Vk02 has a coding region which is indistinguishable from that of vk012 and therefore the assignment of the antibody light chains to either germline gene is equally valid. The light chains of 6F and 10I share 98.9% and 99.6% nucleotide identity, respectively, with the vk01/012 germline gene. Two other anti-TPO antibodies (2G4 and 7F) use light chain genes which show greatest homology, 87 and 97%, respectively, to the kv325 germline gene (Radoux et al., J. Exp. Med., 164:2119-2124 (1986)). The kv325 germline gene is also described in this invention as a universal light chain and is the light chain sequence given in SEQ ID NO 2.

To address the question of bias in the light chain representation in the thyroid peroxidase antibody library, eleven clones were randomly selected from the library before antigen selection and the nucleotide sequence determined. The data indicates

that all eleven light chain sequences are different from each other and from the anti-TPO Fab light chain amino acid residue sequences. The eleven clones were derived from three different kappa gene families, indicating a diverse library. Analysis of the eleven sequences revealed that 2 (1%) used vk02/012 and that 4 (36%) used kv325 which are similar frequencies to those obtained in the anti-gp120 antibodies also described herein. Given that the vk02/012 and kv325 genes constitute only 3 out of the 45-50 germline kappa genes, it appears that these genes are present at a higher than expected frequency in the unselected library. This could be due to bias introduced by the design of the PCR primers, however, the vk02/012 germline light chain is also represented strongly in the SP series of anti-TPO antibodies which were derived using different PCR primers. In addition, the vk02/012 and kv325 light chains are frequently represented in human hybridoma derived antibodies against several non-self antigens. This could be interpreted as an over representation of the vk02/012 and kv325 light chains in antibody-producing cells in both normal and autoimmune cells.

The native light chains of two of the antibodies, 6F and 10I, use the same germline Vkappa gene, vk02/012, as do the SP family of anti-TPO autoantibodies. The vk02/012 gene is also expressed in several other autoantibodies including acetylcholine receptor autoantibodies and rheumatoid factors.

Light and heavy chain pairs derived from hybridomas represent an in vivo pairing while recombinant antibodies produced as described herein may represent both the in vivo and in vitro pairings. To determine the frequency of occurrence of the

vk02/012 and vk325 light chains in known light chain sequences, the nucleotide sequence database was searched with the germline variable region encoding sections of vk02/012 and vk325. Five out of seven human hybridoma antibodies of known specificity which contain the kv02/012 light chain were autoantibodies. Nineteen out of 24 antibodies with the kv325 light chain of known specificity recognized were autoantibodies. The hybridoma antibodies against non-self antigens displayed a wide range of specificities, including Haemophilus influenzae (kv02/012), hepatitis B virus (kv02/012), Neisseria meningitides (kv325), human cytomegalovirus (kv325), and HIV (kv325). Thus, in vivo pairings, as represented by hybridoma antibodies, also contain a high frequency of the kv02/012 and kv325 light chain. Further, in a diverse, non-antigen selected sample of 34 kappa light chain genes, amplified from peripheral blood lymphocytes by PCR (Marks et al., Eur. J. Immunol., 21:985-991 (1991)), vk02/012 was represented four times. In previous studies on murine responses against the hapten NPN and in the human response against HIV-1 gp120 protein, considerable promiscuity of pairing of light chains with a particular heavy chain has been observed. Taken together, and given the over representation of autoantibodies in the database, these results indicate that expression of the k02/012 light chain gene is high, not only in autoimmune but also in normal immune responses. The vk02/012 may therefore be a much-used "plastic" light chain, or a "universal" light chain, which can combine with different heavy chains where specificity is dictated by the heavy chain.

The native light chain in the pC3AP313 phagemid expression vector that binds to tetanus toxoid, kv325,

has been identified in antibodies against foreign antigens such as cytomegalovirus and digoxin. With the methodology of repertoire cloning and sequencing, the pC3AP313 light chain has been observed with a high frequency. For example, the light chain was found in the unmutated gene in an antibody binding hepatitis B surface antigen and was slightly mutated in an anti-thyroglobulin antibody. Comparison of 33 antibodies binding to HIV-1 surface glycoprotein gp120 showed that no less than 13 of the antibodies had the pC3AP313 light chain as the closest light chain germline gene.

Thus, the native pC3AP313 light chain and native 6F light chain, have been coined universal light chains due to their high representation in Fab antibody heterodimers obtained through repertoire cloning. The pC3AP313 and 6F light chains are the human germ-line genes Humkv325 and Humkv02/012, respectively, and behave as a universal light chain V region in combination with various J regions in pairing with a wide range of different heavy chain Fab fragments. The light chains thus exhibit plastic behavior in that if in combination with heavy chains that bind to a wide variety of antigens, the specificity and affinity is not abrogated by the presence of the universal light chain. The amino acid residue light chain sequence is unique in this respect and therefore plays an important role in the utility of recombinant antibody libraries from natural and synthetic sources.

The ability to produce human anti-hapten antibodies that have either the native pC3AP313 encoded universal light chain sequence or further randomized to improve the specificity and affinity of the heterodimer binding may be significant in the

development of catalytic antibodies as
pharmaceuticals. Moreover, the ability to generate
unique crossed libraries having native/native heavy
and light chain CDR domains, native heavy and
5 randomized light chain CDR domains, randomized heavy
and native light chain CDR domains, and finally both
randomized heavy and light chain CDR domains is a
valuable methodology provided by this invention to
create new and improved Fab heterodimers with new or
10 improved specificities and affinities through
expression of selected clones from the libraries.

10. Deposit of Materials

The following plasmid was deposited on or before
15 February 2, 1993, with the American Type Culture
Collection, 1301 Parklawn Drive, Rockville, MD, USA
(ATCC):

<u>Material</u>	<u>ATCC Accession No.</u>
20 Plasmid pC3AP313	ATCC 75408

This deposits was made under the provisions of
the Budapest Treaty on the International Recognition
of the Deposit of Microorganisms for the Purpose of
25 Patent Procedure and the Regulations thereunder
(Budapest Treaty). This assures maintenance of a
viable plasmid deposit for 30 years from the date of
deposit. The deposit will be made available by ATCC
under the terms of the Budapest Treaty which assures
30 permanent and unrestricted availability of the progeny
of the viable plasmids to the public upon issuance of
the pertinent U.S. patent or upon laying open to the
public of any U.S. or foreign patent application,
whichever comes first, and assures availability of the
35 progeny to one determined by the U.S. Commissioner of

Patents and Trademarks to be entitled thereto according to 35 U.S.C. §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the plasmid deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same plasmid. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the plasmid deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any plasmid vectors that are functionally equivalent are within the scope of this invention. The deposit of material does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Barbas, Carlos F,III
Burton, Dennis R
Lerner, Richard A
- (ii) TITLE OF INVENTION: METHODS FOR PRODUCING ANTIBODY LIBRARIES
USING UNIVERSAL OR RANDOMIZED IMMUNOGLOBULIN LIGHT CHAINS
- (iii) NUMBER OF SEQUENCES: 70
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: The Scripps Research Institute
(B) STREET: 10666 North Torrey Pines Road, TPC8
(C) CITY: La Jolla
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92037
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 02-SEP-1994
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/174,674
(B) FILING DATE: 28-DEC-1993
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/826,623
(B) FILING DATE: 27-JAN-1992
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/954,148
(B) FILING DATE: 30-SEP-1992
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/012,566
(B) FILING DATE: 02-FEB-1993
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Fitting, Thomas
(B) REGISTRATION NUMBER: 34,163

005040-07500

(C) REFERENCE/DOCKET NUMBER: TSRI 409.1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 619-554-2937

(B) TELEFAX: 619-554-6312

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 687 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGAGCAGT CTGGGGCTGA GGTGAAGAAG CCTGGGTCCT CGGTGAAGGT CTCCTGCAGG 60
GCTTCTGGAG GCACCTTCAA CAATTATGCC ATCAGCTGGG TGCACAGGC CCCTGGACAA 120
GGGCTTGAGT GGATGGGAGG GATCTTCCCT TTCCGTAATA CAGCAAAGTA CGCACAACAC 180
TTCCAGGGCA GAGTCACCAT TACCGCGGAC GAATCCACGG GCACAGCCTA CATGGAGCTG 240
AGCAGCCTGA GATCTGAGGA CACGGCCATA TATTATTGTG CGAGAGGGGA TACGATTTTT 300
GGAGTGACCA TGGGATACTA CGCTATGGAC GTCTGGGGCC AAGGGACCAC GGTACCGTC 360
TCCGCAGCCT CCACCAAGGG CCCATCGGTC TTCCCCCTGG CACCCTCCTC CAAGAGCACC 420
TCTGGGGGCA CAGCGGCCCT GGGCTGCCTG GTCAAGGACT ACTTCCCCGA ACCGGTGACG 480
GTGTCGTGGA ACTCAGGCGC CCTGACCAGC GGC GTGCACA CCTTCCCGGC TGTCTACAG 540
TCCTCAGGAC TCTACTCCCT CAGCAGCGTG GTGACCGTGC CCTCCAGCAG CTTGGGCACC 600
CAGACCTACA TCTGCAACGT GAATCACAAG CCCAGCAACA CCAAGGTGGA CAAGAAAGCA 660
GAGCCCAAAT CTTGTGACAA AACTAGT 687

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 646 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAGCTCACGC AGTCTCCAGG CACCCTGTCT TTGTCTCCAG GGGAAAGAGC CACCCTCTCC	60
TGCAGGGCCA GTCACAGTGT TAGCAGGGCC TACTTAGCCT GGTACCAGCA GAAACCTGGC	120
CAGGCTCCCA GGCTCCTCAT CTATGGTACA TCCAGCAGGG CCACTGGCAT CCCAGACAGG	180
TCCAGTGGCA GTGGGTCTGG GACAGACTTC ACTCTCACCA TCAGCAGACT GGAGCCTGAA	240
GATTTTGAG TGTACTACTG TCAGCAGTAT GGTGGCTCAC CGTGGTTCGG CCAAGGGACC	300
AAGGTGGAAC TCAAACGAAC TGTGGCTGCA CCATCTGTCT TCATCTTCCC GCCATCTGAT	360
GAGCAGTTGA AATCTGGAAC TGCCTCTGTT GTGTGCCTGC TGAATAACTT CTATCCCAGA	420
GAGGCCAAAG TACAGTGGAA GGTGGATAAC GCCCTCCAAT CGGGTAACTC CCAGGAGAGT	480
GTCACAGAGC AGGACAGCAA GGACAGCACC TACAGCCTCA GCAGCACCCT GACGCTGAGC	540
AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA GGGCCTGACT	600
TCGCCCCGTC CAAAGAGCTT CAACAGGGGA GAGTGTAAAT TCTAGA	646

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

00540541-070500

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCTAAA CTAGCTAGTC G

21

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATACTGCTGA CAGTAATACA C

21

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 57 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TATTACTGTC AGCAGTATNN KNNKNNKNNK ACTTTCGGCG GAGGGACCAA GGTGGAG

57

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

005020-15507560

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATACGACTC ACTATAGGGC G

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TATTACTGTC AGCAGTATNN KNNKNNKNNK ACTTTCGGCG GAGGGACC

48

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TATTACTGTC AGCAGTATNN KNNKNNKNNK NNKACTTTCG GCGGAGGGAC CAAGGTGGAG

60

005040-030500

(2) INFORMATION FOR SEQ ID NO:10:

- (iv) ANTI-SENSE: NO

TATTACTGTC AGCAGTATNN KNNKNNKNNK NNACTTTTCG GCGGAGGGAC C

(i) SEQUENCE CHARACTERISTICS:

- (iv) ANTI-SENSE: NO

GATTTTGCAG TGTATTACTG TCAGCAGTAT NNKNNKNNKN NKNNKNNKAC TTTCGGCGGA 60

GGGACCAAGG TGGAG 75

(i) SEQUENCE CHARACTERISTICS:

- (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TATTACTGTC AGCAGTATNN KNNKNNKNNK NNNKNNKACTT TCGGCGGAGG GACC

54

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GATTTTGCAG TGTATTACTG TNNKNNKNNK NNNKNNKNNK NNNKNNKNNK KTTTCGGCGGA

60

GGGACCAAGG TGGAG

75

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTCCACCTT GGTCCCTTGG CCGAAMNNMN NMNNMNNMNN MNNMNNMNNNA CAGTAGTACA

60

005020-1590460

CTGCAAAATC

70

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 76 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTCCACCTT GGTCCCTTGG CCGAAMNNMN NMNNMNNMNN MNNMNNMNNM NNMNNACAGT 60

AGTACACTGC AAAATC 76

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 94 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTCCACCTT GGTCCCTTGG CCGAAMNNMN NMNNMNNMNN MNNMNNMNNM NNMNNMNNMN 60

NMNNMNNMNN MNNACAGTAG TACACTGCAA AATC 94

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

00540541-020500

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTCGGCCAAG GGACCAAGGT GGAAC

25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCAATTAACC CTCACTAAAG GG

22

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

005020-150750

TCTCGCACAG TAATACACGG CCGT

24

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 57 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCCGTGTATT ACTGTGCGAG ANNKNNKNNK GACNNKTGGG GCCAAGGGAC CACGGTC

57

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTGATATTCA CAAACGAATG G

21

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

005040-1507500

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCCGTGTATT ACTGTGCGAG AGGTNNKNNK NNKNNKNNKN NKNNKGACNN KTGGGGCCAA 60

GGGACCACGG TC 72

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 90 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCCGTGTATT ACTGTGCGAG AGGTNNKNNK NNKNNKNNKN NKNNKNNKNN KNNKNNKNNK 60

NNKGACNNKT GGGGCCAAGG GACCACGGTC 90

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

004091-0900

GTGTATTATT GTGCGAGANN SNNSNNSNNS NNSTGGGGCC AAGGGACCAC G

51

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 66 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTGTATTATT GTGCGAGANN SNNSNNSNNS NNSNNSNNSN NSNNSNNSTG GGGCCAAGGG

60

ACCACG

66

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 84 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTGTATTATT GTGCGAGANN SNNSNNSNNS NNSNNSNNSN NSNNSNNSNN SNNSNNSNNS

60

NNSNNSSTGGG GCCAAGGGAC CACG

84

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TATACTGTCA GCAGTAT

17

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GATTTTGCAG TGTATTACTG TCAGCAGTAT

30

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

005020.7507.960

ACTTTCGGCG GAGGGACCAA GGTGGAG

27

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACTTTCGGCG GAGGGACC

18

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTTCCACCTT GGTCCCTTGG CCGAA

25

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

005020-15501960

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ACAGTAGTAC ACTGCAAAAT C

21

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Gly Trp Ser Arg Trp Ser Gly Leu Asp Trp
1 5 10

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ser Ser Thr Lys Ile Met Arg Leu Asp Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

005020-15507900

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Gly	Met	Phe	Arg	Arg	Gly	Phe	Tyr	Asp	Arg
1				5				10	

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Gly	Val	Arg	Asn	Asn	Phe	Gly	Arg	Trp	His	Trp	Val	Trp	Asp	Ser
1				5				10					15	

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Gly	Arg	Ala	Val	Arg	Gly	Ser	Arg	Lys	Arg	Val	Leu	Gly	Tyr	Asp	Arg
1				5				10						15	

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

00502075507960

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Gly	Arg	Pro	Gly	Val	Val	Arg	Arg	Arg	Ile	Ala	Pro	Arg	Met	Asp	Ile
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gly	Pro	Lys	Gly	Val	Phe	Pro	Arg	Trp	Gly	Met	Ala	Ser	Phe	Asp	Arg
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Gly	Val	Asn	Leu	Phe	Arg	Val	Arg	Asn	Ser	Arg	Pro	His	Leu	Asp	Met
1				5					10					15	

005020-070500

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Gly	Leu	Arg	Gly	Ser	Arg	Gly	Phe	Asp	Arg
1				5					10

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Gly	Ser	Trp	Leu	Arg	Gly	Pro	Tyr	Asp	Met
1				5					10

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Gly Thr Leu Gly Glu Gly Gly Tyr Asp Arg

005040-15501960

1 5 10

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Gly Trp Arg Ser Ser Arg Gly Val Val Trp Val Phe Ser Gly Asp Ala
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Gly Asp Trp Gly Trp Phe Thr Arg Val Ala Thr Trp Arg Pro Asp Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

005020-7507960

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Gln Gln Tyr Leu Pro Gly Gly Arg Tyr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Gln Gln Tyr Arg Val Glu Gly Gln Thr
1 5

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Gln Gln Tyr Gly Gly Ser Pro Trp
1 5

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

005020-750750

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Gln Gln Tyr Ser Arg His Arg Phe Thr
1 5

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Gln Gln Tyr Arg Tyr Pro Leu Ile Trp Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Gln Gln Tyr Gly Ser Ser Leu Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

005040-1507960

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Gln Gln Tyr Thr Arg Pro Gly Val Thr
1 5

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Gln Gln Tyr Ser Phe Lys Asn Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Gln Gln Tyr Gly Tyr Arg Lys Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

005029-00500

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gln	Gln	Tyr	Thr	Pro	Arg	Arg	Gly	Ala	Thr
1				5					10

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gln	Gln	Tyr	Thr	Pro	Arg	Val	Gly	His	Thr
1				5					10

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Gln	Gln	Tyr	Lys	Tyr	Gly	Arg	Gly	Met	Thr
1				5					10

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

005020-1950F960

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Gln Gln Tyr Gly Lys Lys Gln Trp Thr
1 5

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Gln Gln Tyr Val Arg Arg Ser Gly Thr
1 5

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Gln Gln Tyr Gly Lys Arg Ser Pro Val Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

005020-15907960

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Gln Gln Tyr Ala Arg Ala Thr Gly Leu Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Gln Gln Tyr Ser Arg Phe Val Ser Arg Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 280 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GAGCTCACCC AGTCTCCATC CTCCCTGTCT GCATCTGTAG GAGACAGAGT CACCATCACT	60
TGCCGGGCAA GTCAGCGCAT TAGCAGCTAT TTAAATTGGT ATCAGCAGGA ACCAGGGGAA	120
CCCCCTAAGC TCCTGATCTA TGCTGCATCC AGGTTTGCAA AGTGGGGTCC CATCAAGGTT	180
CAGTGGCAGT GGATCTGGGA CAGATTTAC TCTCACCATC AGCAGTCTGC AACCTGAAGA	240

TTTTGCAACT TACTACTGTC AACAGAGTTA CAGTACCCCG

280

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 124 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Leu	Glu	Ser	Gly	Gly	Asp	Leu	Val	Gln	Pro	Gly	Gly	Ser	Leu	Arg	Leu	1	5	10	15
Ser	Cys	Glu	Ala	Ser	Gly	Phe	Thr	Phe	Gly	Ser	Tyr	Ala	Met	Thr	Trp	20	25	30	
Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val	Ser	Ser	Pro	Ser	35	40	45	
Ala	Asn	Gly	Asp	Phe	Ala	Tyr	Tyr	Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	50	55	60	
Thr	Ile	Ser	Arg	Asp	Lys	Ser	Lys	His	Thr	Leu	Phe	Leu	Gln	Met	His	65	70	75	80
Ser	Leu	Arg	Val	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Lys	Ala	Gly	85	90	95	
Arg	Ile	Leu	Gly	Val	Val	Leu	Trp	Tyr	Ser	Leu	Tyr	Tyr	Gly	Phe	Asp	100	105	110	
Val	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser	115	120						

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 118 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

0059022-15501960

Leu Glu Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys
1 5 10 15

Val Ser Cys Lys Ala Ser Gly Tyr Asn Phe Asn Ser His Asp Ile Asn
20 25 30

Trp Val Arg Gln Ala Thr Gly Gln Gly Leu Glu Trp Ile Gly Trp Ile
35 40 45

Thr Asn Arg Gly Thr Thr Ser Arg Tyr Ala Gln Lys Phe Gln Gly Arg
50 55 60

Val Thr Met Thr Arg Asp Ala Ser Ile Ser Thr Val Tyr Met Glu Leu
65 70 75 80

Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Gly
85 90 95

Ala Gly Ala Gly Gly Thr Trp Gly Met Asp Val Trp Gly Gln Gly Thr
100 105 110

Thr Val Ile Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Gln Val Lys Leu Leu Glu Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Gly His
20 25 30

Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Trp Ile Ser Pro Asn Arg Gly Ala Thr Arg Phe Ala Gln Lys Phe
50 55 60

Gln Gly Arg Val Thr Met Thr Ser Asp Thr Ser Ile Asn Thr Val Tyr
65 70 75 80

005920-15901360

Met Glu Leu Ser Gly Leu Arg Phe Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Thr Thr Arg Thr Ala Tyr Tyr Gly Met Asp Val Trp Gly Gln Gly
100 105 110

Thr Thr Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Glu Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
1 5 10 15

Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Tyr Ile Asn
20 25 30

Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ala
35 40 45

Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly
50 55 60

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
65 70 75 80

Phe Ala Thr Tyr Tyr Cys Gln Gln Ser Tyr Ser Thr Pro Phe Thr Phe
85 90 95

Cys Pro Gly Thr Lys Val Asp Ile Lys Arg Thr
100 105

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

005040-7507500

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Glu	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly	Asp	Arg	1	5	10	15
Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Arg	Ile	Ser	Ser	Tyr	Ile	Asn	20	25	30	
Trp	Tyr	Gln	Gln	Glu	Lys	Pro	Gly	Ala	Pro	Lys	Leu	Leu	Ile	Tyr	Ala	35	40	45	
Ala	Ser	Ser	Leu	Gln	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	50	55	60	
Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro	Glu	Asp	65	70	75	80
Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Ser	Tyr	Ser	Thr	Pro	Tyr	Thr	Phe	85	90	95	
Cys	Gln	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	Thr	100	105							

(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 109 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Glu	Leu	Val	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Glu	Gly	1	5	10	15
Asp	Thr	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Glu	Asn	Ile	Ser	Arg	Tyr	20	25	30	
Ser	Asn	Trp	Tyr	Gln	Gln	Gln	Pro	Gly	Lys	Ala	Pro	Lys	Leu	Leu	Ile	35	40	45	
Ser	Ala	Ala	Ser	Thr	Leu	Gln	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	50	55	60	
Ser	Gly	Ser	Gly	Thr	His	Phe	Thr	Leu	Thr	Ile	Asn	Ser	Leu	Gln	Pro	65	70	75	80

006409.1-020600

Gly Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Thr Tyr Ser Ser Pro Phe
85 90 95

Thr Phe Cys Gln Gly Thr Lys Leu Glu Ile Lys Arg Thr
100 105

(2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 109 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Glu Leu Val Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Thr Ile Gly Thr Tyr
20 25 30

Ile Asn Trp Tyr Gln Gln Lys Pro Gly Glu Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Thr Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Arg Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ser Tyr Ser Thr Pro Trp
85 90 95

Thr Phe Cys Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
100 105

(2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 110 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

005020-1507950

Glu	Leu	Val	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly	1	5	10	15
Asp	Arg	Val	Thr	Ile	Ser	Gly	Cys	Arg	Ala	Ser	Gln	Asn	Ile	Gly	Lys	20	25	30	
Tyr	Ile	Asn	Trp	Tyr	Arg	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Glu	Leu	Leu	35	40	45	
Ile	Tyr	Gly	Thr	Ser	Thr	Leu	Gln	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	50	55	60	
Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	65	70	75	80
Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Ser	Tyr	Ser	Thr	Pro	85	90	95	
Trp	Thr	Phe	Cys	Gln	Gly	Thr	Lys	Val	Glu	Ile	Lys	Arg	Thr	100	105	110			

005040-030500

What Is Claimed Is:

1. An oligonucleotide useful as a primer for inducing mutagenesis in a complementarity determining region (CDR) of an immunoglobulin light chain gene, said oligonucleotide having 3' and 5' termini and comprising:

a) a nucleotide sequence at said 3' terminus capable of hybridizing to a first framework region of an immunoglobulin gene;

b) a nucleotide sequence at said 5' terminus capable of hybridizing to a second framework region of an immunoglobulin gene; and

c) a nucleotide sequence between said 3' and 5' termini according to the formula:

$[NNK]_n$,

wherein N is independently any nucleotide, K is G or T, n is 3 to about 24, said 3' and 5' terminal nucleotide sequences having a length of about 6 to 50 nucleotides, or an oligonucleotide having a sequence complementary thereto.

2. The oligonucleotide of claim 1 wherein said 5' terminus has the nucleotide sequence 5'-TATACTGTCAGCAGTAT-3' (SEQ ID NO 26) or 5'-GATTTTGCAGTGTATTACTGTCAGCAGTAT-3' (SEQ ID NO 27), or an oligonucleotide having a sequence complementary thereto.

3. The oligonucleotide of claim 1 wherein said 3' terminus has the nucleotide sequence 5'-ACTTTCGGCGGAGGGACCAAGGTGGAG-3' (SEQ ID NO 28) or 5'-ACTTTCGGCGGAGGGACC-3' (SEQ ID NO 29), or an oligonucleotide having a sequence complementary thereto.

4. The oligonucleotide of claim 1 wherein n is 4, 5, 6, 10 or 16.

5. The oligonucleotide of claim 1 wherein said immunoglobulin is human.

6. The oligonucleotide of claim 1 wherein said CDR is CDR3.

5 7. The oligonucleotide of claim 1 according to the formula: 5'-

GATTTTGCAGTGTATTACTGT[NNK]₁₀TTCGGCGGAGGGACCAAGGTGGAG-
3' (SEQ ID NO 12), or an oligonucleotide having a
sequence complementary thereto.

10 8. An oligonucleotide useful as a primer for inducing mutagenesis in a complementarity determining region (CDR) of an immunoglobulin light chain gene, said oligonucleotide having 3' and 5' termini and comprising:

15 a) a nucleotide sequence at said 3' terminus capable of hybridizing to a first framework region of an immunoglobulin gene;

b) a nucleotide sequence at said 5' terminus capable of hybridizing to a second framework
20 region of an immunoglobulin gene; and

c) a nucleotide sequence between said 3' and 5' termini according to the formula:

[MNN]_n,

25 wherein N is independently any nucleotide, M is A or C, n is 3 to about 24, said 3' and 5' terminal nucleotide sequences having a length of about 6 to 50 nucleotides, or an oligonucleotide having a sequence complementary thereto.

30 9. The oligonucleotide of claim 8 wherein said 5' terminus has the nucleotide sequence 5'-
GTTCCACCTTGGTCCCTTGGCCGAA-3' (SEQ ID NO 30), or an oligonucleotide having a sequence complementary thereto.

35 10. The oligonucleotide of claim 8 wherein said 3' terminus has the nucleotide sequence 5'-

ACAGTAGTACACTGCAAAATC-3' (SEQ ID NO 31), or an oligonucleotide having a sequence complementary thereto.

11. The oligonucleotide of claim 8 wherein n is 8, 10 or 16.

12. The oligonucleotide of claim 8 wherein said immunoglobulin is human.

13. The oligonucleotide of claim 8 wherein said CDR is CDR3.

14. A method for producing an antibody combining site in a polypeptide comprising inducing mutagenesis in a complementarity determining region (CDR) of an immunoglobulin light chain gene which comprises amplifying a CDR portion of the immunoglobulin gene by polymerase chain reaction (PCR) using a PCR primer oligonucleotide, said oligonucleotide having 3' and 5' termini and comprising:

a) a nucleotide sequence at said 3' terminus capable of hybridizing to a first framework region of an immunoglobulin gene;

b) a nucleotide sequence at said 5' terminus capable of hybridizing to a second framework region of an immunoglobulin gene; and

c) a nucleotide sequence between said 3' and 5' termini according to the formula:

$[NNK]_n$,

wherein N is independently any nucleotide, K is G or T, and n is 3 to about 24, said 3' and 5' terminal nucleotide sequences having a length of about 6 to 50 nucleotides, or an oligonucleotide having a sequence complementary thereto.

15. The method of claim 14 wherein said 5' terminus has the nucleotide sequence 5'-

TATACTGTCAGCAGTAT-3' (SEQ ID NO 26) or 5'-

GATTTTGCAGTGTATTACTGTCAGCAGTAT-3' (SEQ ID NO 27), or

an oligonucleotide having a sequence complementary thereto.

16. The method of claim 14 wherein said 3' terminus has the nucleotide sequence 5'-
5 ACTTTCGGCGGAGGGACCAAGGTGGAG-3' (SEQ ID NO 28) or 5'-
ACTTTCGGCGGAGGGACC-3' (SEQ ID NO 29), or an oligonucleotide having a sequence complementary thereto.

17. The method of claim 14 wherein n is 4, 5, 6,
10 10 or 16.

18. The method of claim 14 wherein said immunoglobulin is human.

19. The method of claim 14 wherein said CDR is CDR3.

20. The method of claim 14 according to the formula: 5'-
15 GATTTTGCAGTGTATTACTGT[NNK]₁₀TTCGGCGGAGGGACCAAGGTGGAG-
3' (SEQ ID NO 12), or an oligonucleotide having a sequence complementary thereto.

21. The method of claim 14 wherein said immunoglobulin light chain gene includes a sequence having the sequence characteristics of the light chain shown in SEQ ID NO 2 or in SEQ ID NO 62.

22. The method of claim 14 wherein said
25 immunoglobulin light chain gene has the sequence characteristics of the light chain gene in ATCC Accession No. 75408.

23. The method of claim 14 that further comprises the steps of:

30 a) isolating the amplified CDR to form a library of mutagenized immunoglobulin light chain genes;

b) expressing the isolated library of mutagenized light chain genes in combination with one
35 or more heavy chain genes to form a combinatorial

antibody library of expressed heavy and light chain genes; and

c) selecting species of said combinatorial antibody library for the ability to bind a preselected antigen.

24. The method of claim 23 wherein said one or more immunoglobulin heavy chain genes is a library of heavy chain genes.

25. A method for producing an antibody combining site in a polypeptide comprising inducing mutagenesis in a complementarity determining region (CDR) of an immunoglobulin light chain gene which comprises amplifying a CDR portion of the immunoglobulin gene by polymerase chain reaction (PCR) using a PCR primer oligonucleotide, said oligonucleotide having 3' and 5' termini and comprising:

a) a nucleotide sequence at said 3' terminus capable of hybridizing to a first framework region of an immunoglobulin gene;

b) a nucleotide sequence at said 5' terminus capable of hybridizing to a second framework region of an immunoglobulin gene; and

c) a nucleotide sequence between said 3' and 5' termini according to the formula:

$$[MNN]_n,$$

wherein N is independently any nucleotide, M is A or C, n is 3 to about 24, said 3' and 5' terminal nucleotide sequences having a length of about 6 to 50 nucleotides, or an oligonucleotide having a sequence complementary thereto.

26. The method of claim 25 wherein said 5' terminus has the nucleotide sequence 5'-GTTCCACCTTGGTCCCTTGGCCGAA-3' (SEQ ID NO 30), or an oligonucleotide having a sequence complementary thereto.

27. The method of claim 25 wherein said 3' terminus has the nucleotide sequence 5'-ACAGTAGTACACTGCAAAATC-3' (SEQ ID NO 31), or an oligonucleotide having a sequence complementary thereto.

28. The method of claim 25 wherein n is 8, 10 or 16.

29. The method of claim 25 wherein said immunoglobulin is human.

30. The method of claim 25 wherein said CDR is CDR3.

31. The method of claim 25 wherein said immunoglobulin light chain gene includes a sequence having the sequence characteristics of the light chain shown in SEQ ID NO 2 or in SEQ ID NO 62.

32. The method of claim 25 wherein said immunoglobulin light chain gene has the sequence characteristics of the light chain gene in ATCC Accession No. 75408.

33. The method of claim 25 that further comprises the steps of:

a) isolating the amplified CDR to form a library of mutagenized immunoglobulin light chain genes;

b) expressing the isolated library of mutagenized light chain genes in combination with one or more heavy chain genes to form a combinatorial antibody library of expressed heavy and light chain genes; and

c) selecting species of said combinatorial antibody library for the ability to bind a preselected antigen.

34. The method of claim 33 wherein said one or more immunoglobulin heavy chain genes is a library of heavy chain genes.

35. A method for producing a heterodimeric immunoglobulin molecule having immunoglobulin variable domain heavy and light chain polypeptides comprising the steps of:

5 a) combining an immunoglobulin variable domain light chain gene that includes a sequence having the sequence characteristics of the light chain shown in SEQ ID NO 2 or 62 with one or more immunoglobulin variable domain heavy chain genes to
10 form a combinatorial immunoglobulin heavy and light chain gene library, said combining comprising operatively linking said light chain gene with one of said heavy chain genes in a vector capable of co-expression of said heavy and light chain genes;

15 b) expressing the combinatorial gene library to form a combinatorial antibody library of expressed heavy and light chain polypeptides; and

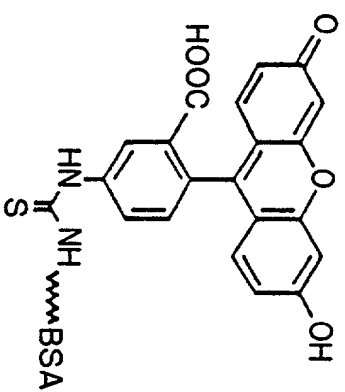
20 c) selecting species of said combinatorial antibody library for the ability to bind a preselected antigen.

36. The method of claim 35 wherein said immunoglobulin light chain gene has the sequence characteristics of the light chain gene in ATCC Accession No. 75408.

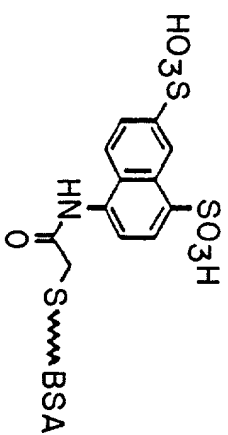
25 37. The method of claim 35 wherein said one or more immunoglobulin heavy chain genes is a library of heavy chain genes.

ABSTRACT

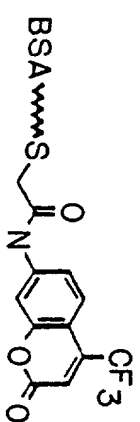
The present invention describes methods for
producing antibody libraries, and particularly for
5 increasing antibody library diversity by inducing
mutagenesis within the CDR regions of immunoglobulin
heavy or light chains that are displayed on the
surface of filamentous phage particles comprising the
library. The invention also describes
10 oligonucleotides useful for increasing the library
diversity, and universal light chains useful in the
library production methods.



1=F I-BSA



2=S-BSA



3=C-BSA

FIG. 1

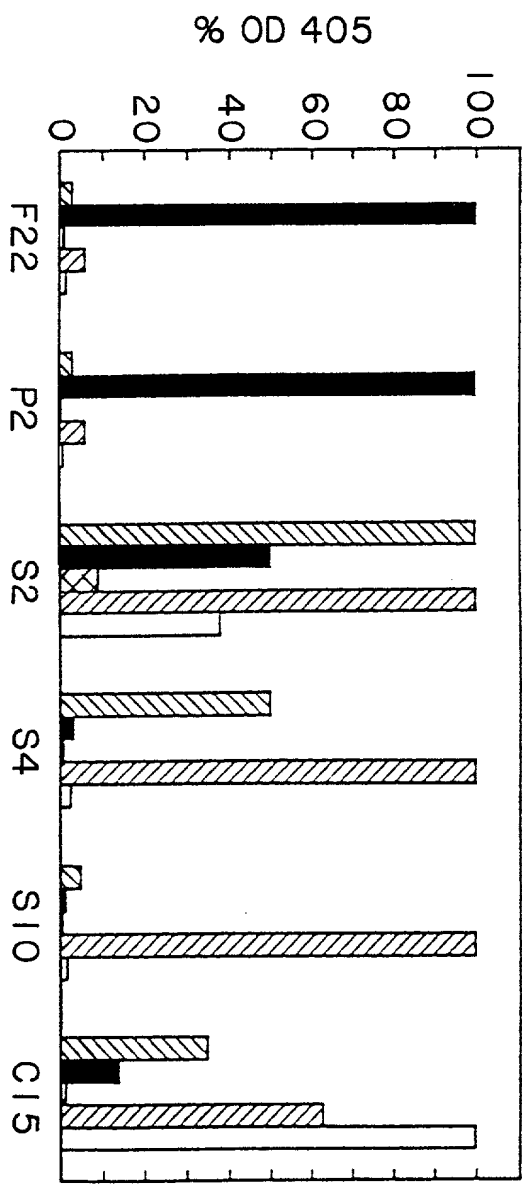


FIG. 2

0964054-070500

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY

I HEREBY DECLARE THAT:

My residence, post office address, and citizenship are as stated next to my name in PART A of page 2 hereof.

I believe I am the original, first, and sole inventor (if only one name is listed) or an original, first, and joint inventor (if plural names are listed) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHODS FOR PRODUCING ANTIBODY LIBRARIES USING
UNIVERSAL OR RANDOMIZED IMMUNOGLOBULIN LIGHT CHAINS

the specification of which:

X is attached hereto

— was filed on _____ as Application Serial No. _____
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Sec. 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Sec. 119 of any foreign application(s) for patent or inventor's certificate listed in PART B on page 2 hereof and have also identified in PART B on page 2 hereof any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

I hereby claim the benefit under Title 35, United States Code, Sec. 120 of any United States application(s) listed in PART C on page 2 hereof and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Sec. 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sec. 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following as my attorneys or agents with full power of substitution to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

Douglas A. Bingham	Reg. No. 32,457	April C. Logan	Reg. No. 33,950
Thomas Fitting	Reg. No. 34,163	Donald G. Lewis	Reg. No. 28,636

whose mailing address for this application is:

THE SCRIPPS RESEARCH INSTITUTE
10666 North Torrey Pines Road
Mail Drop TPC 8
La Jolla, California 92037

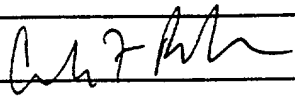
See Page 2 attached, signed, and made a part hereof.

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY

PART A: Inventor Information And Signature


Full name of SOLE or FIRST inventor Carlos F. Barbas
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Residence (if different) _____

Inventor's Signature:  Date: 8/24/94

Full name of SECOND joint inventor, if any Dennis R. Burton
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Residence (if different) _____

Second Inventor's Signature:  Date: Aug 24 94

Full name of THIRD joint inventor, if any Richard A. Lerner
Citizenship USA Post Office Address 7750 E. Roseland Drive
La Jolla, California 92037

Residence (if different) _____

Third Inventor's Signature:  Date: 9-1-94

Full name of FOURTH joint inventor, if any _____
Citizenship _____ Post Office Address _____

Residence (if different) _____

Fourth Inventor's Signature: _____ Date: _____

Full name of FIFTH joint inventor, if any _____
Citizenship _____ Post Office Address _____

Residence (if different) _____

Fifth Inventor's Signature: _____ Date: _____

PART B: Prior Foreign Application(s)

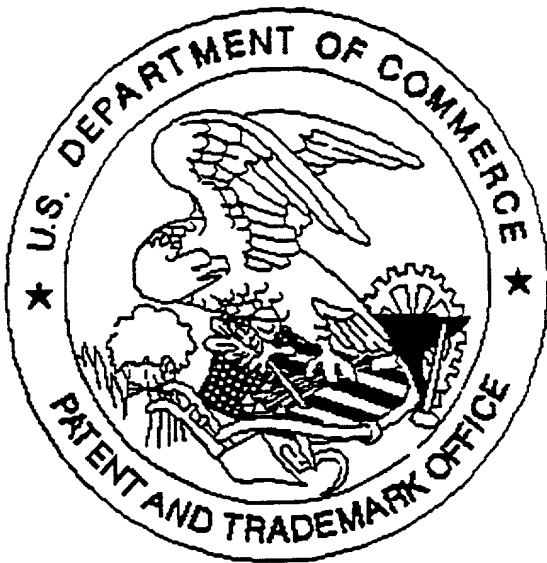
Serial No.	Country	Day/Month/Year Filed	Priority Claimed
			<input type="checkbox"/> Yes <input type="checkbox"/> No
			<input type="checkbox"/> Yes <input type="checkbox"/> No

PART C: Claim For Benefit of Filing Date of Earlier U.S. Application(s)

Serial No.	Filing Date	Status:
07/826,623	1/27/92	<input type="checkbox"/> Patented <input checked="" type="checkbox"/> Pending <input type="checkbox"/> Abandoned
07/954,148	9/30/92	<input type="checkbox"/> Patented <input type="checkbox"/> Pending <input checked="" type="checkbox"/> Abandoned
08/012,566	2/2/93	<input type="checkbox"/> Patented <input type="checkbox"/> Pending <input checked="" type="checkbox"/> Abandoned
08/174,674	12/28/94	<input type="checkbox"/> Patented <input checked="" type="checkbox"/> Pending <input type="checkbox"/> Abandoned

See Page 1 to which this is attached and from which this Page 2 continues.

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declaration

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